

## ABSTRACT

VOGEL III, KENNETH GEORGE. Protein Amount and Milk Protein Ingredient Effects on Sensory and Physicochemical Properties of Ready-To-Drink Protein Beverages. (Under the direction of Dr. MaryAnne Drake).

This study evaluated the role of protein concentration and milk protein ingredient (serum protein isolate (SPI), micellar casein (MCC), or milk protein concentrate (MPC)) on sensory and physicochemical properties of vanilla ready-to-drink (RTD) protein beverages. The RTD beverages were manufactured from 5 different liquid milk protein blends (100% MCC, 100% MPC, 18:82 SPI:MCC, 50:50 SPI:MCC, and 50:50 SPI:MPC) at 2 different protein concentrations 6.3% and 10.5% protein (15 or 25 g protein per 237 mL) with 0.5% w/w fat and 0.7% w/w lactose. Dipotassium phosphate, carrageenan, cellulose gum, sucralose and vanilla flavor were included. Blended beverages were pre-heated to 65°C and homogenized (20.7 MPa) and cooled to 8°C. The beverages were then preheated to 92°C, and then ultrapasteurized (141°C, 3 sec) followed by vacuum cooling to 92°C and homogenization again (17.2 MPa first stage, 3.5 MPa second stage). Beverages were then cooled, filled into sanitized bottles and stored at 4°C. Initial testing of RTD beverages included proximate analyses and aerobic plate count and coliform count. Volatile sulfur compounds, vanillin, particle size, and sensory properties were evaluated through 8 weeks. Astringency and viscosity were higher and sweet aromatic/vanillin flavor was lower in beverages containing 10.5% protein compared to 6.3% protein ( $P<0.05$ ) and sulfur/eggy flavor, astringency and viscosity were higher and sweet aromatic/vanillin flavor were lower in beverages with higher serum protein as a percentage of true protein within each protein content ( $P<0.05$ ). Volatile compound analysis of headspace vanillin and sulfur compounds was consistent with sensory results, beverages with 50% serum protein as percentage of true protein and 10.5% protein had the highest concentrations of sulfur volatiles and lower vanillin compared

to other beverages ( $P < 0.05$ ). Sulfur volatiles and sulfur/eggy flavors and vanillin and sweet aromatic/vanillin flavor decreased in all beverages with storage time ( $P < 0.05$ ). Particle size was larger in beverages with higher serum protein as a proportion of true protein and the particle size increased with storage time ( $P < 0.05$ ). These results will enable manufacturers to select and or optimize protein blends to better formulate RTD beverages to provide consumers with a protein beverage with high protein content and desired flavor and functional properties.

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Protein Amount and Milk Protein Ingredient Effects on Sensory and Physicochemical Properties  
of Ready-To-Drink Protein Beverages

by  
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## **BIOGRAPHY**

Kenneth Vogel III was born in Illinois to Kenneth Vogel Jr and Dr. Margaret Poole and is the younger of their two children. Ken grew up in Topsfield, Massachusetts where his interest in food science was sparked by a high school science fair project which advanced to the state finals. He later attended The Pennsylvania State University where he continued to explore food science, winning a national product development competition and the IFT college bowl competition. During this time he also worked as a R&D intern at Mars Chocolate North America where he was responsible for Snickers related research. Upon graduating from Penn State in May 2015 with a B.S. in Food Science, he began working at Leprino Foods in Denver, Colorado where he developed the company's first consumer product launched under the Ascent brand. This product would later bring him to North Carolina State University to perform consumer testing, which is where he met Dr. MaryAnne Drake. In June 2016 would then begin to pursue a M.S. degree in Food Science under the direction of Dr. MaryAnne Drake. Outside of school, Ken enjoys exploring local breweries and competing in trivia competitions.

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## CHAPTER 1. LITERATURE REVIEW

### 1. INTRODUCTION

Consumers continue to seek out products that offer benefits for their health and wellness. The 2016 *Food and Health Survey* indicated that 76% shoppers ages 18-80 y in the United States made small changes in their diet throughout the year to achieve an overall healthier diet. Protein was the most sought after nutrient with 64% of respondents indicating they were trying to consume more protein in their diet (The International Food Information Council, 2016). The convenience and variety of ready to drink protein beverages attracts new consumers who are seeking out ways to increase their protein intake. Dairy proteins, specifically milk and whey proteins, are the most desirable types of protein in RTD beverages and higher amounts of protein per serving were also favored (Agarwal et al., 2015; Will S Harwood and Drake, 2019; Oltman et al., 2015). RTD beverages currently on the market have a broad range of protein contents from 10 to 40 g of protein per serving. The variability of protein per serving can partially be attributed to inconsistencies in serving sizes which range from 236 to 591 mL. Even taking into account increases in serving size to increase protein per serving, manufacturers are also increasing concentration of protein in beverages which presents greater technical challenges to formulation and manufacture of these products.

Ready to Drink beverages are divided into two main categories, neutral pH beverages and acidified beverages. Neutral beverages have a pH between 4.6 and 7.5 and acidified beverages ideally have a pH of between 2.5 and 3.0 (Etzel, 2004; Rittmanic and Burrington, 2006). The pH of the beverages determines the thermal treatment the beverages will require to produce a shelf stable product. A low pH (<4.6) reduces the heat resistance of bacteria and other microorganisms (Palop and Martínez, 2005). High acid, low pH, beverages are hot filled, where the beverages are

heated to at least 83 °C (target temperature 90 – 95 °C), then filled into a container and inverted while still hot (Frederick et al., 2010; Rushing and Foegeding, 2010). Neutral pH beverages are required to undergo either retort processing (115 – 125 °C for about 10 – 40 min), or Ultra High Temperature (UHT) treatment (135 – 150 °C with a hold time of 2 – 6 s) followed by cooling and filling aseptically into separately sterilized containers. An understanding of the physicochemical properties of casein and whey proteins is critical to producing a beverage with the desired nutritional profile, functionality and sensory properties.

## 2. OVERVIEW OF DAIRY PROTEINS

Research surrounding milk proteins began as early as 1814 with research by Jöns Jacob Berzelius (1814). While the term “protein” would not be coined until 1838 again by Jöns Jacob Berzelius (Hartley, 1951). Even in recent publications and literature the credit for naming of protein is commonly given to Gerardus Johannes Mulder (O’Mahony and Fox, 2013, 2014). Although Mulder can be credited with the first published use of the term “protein” he did so after seeing the term used in correspondence from Berzelius (Hartley, 1951; Mulder, 1838). A method of separating caseins was developed in 1830 by H. Braconnot who had also introduced the term “casein”. The method of acid precipitation of casein at pH 4.6 was advanced by Olof Hammarsten in 1883 and then again later refined by Lucius L. Van Slyke and John C. Baker (Hammarsten, 1883; Van Slyke and Baker, 1918).

Albumin and globulin fractions of milk protein were discovered by John Sebelein in 1885 by fractionating the liquid remaining after casein precipitation into soluble and insoluble proteins via the addition of MgSO<sub>4</sub>. In 1938, Samuel Rowland further fractionated the remaining nitrogenous components of the milk serum by identifying proteose peptones and non-protein nitrogen using combinations of heat denaturation, acid precipitation, and precipitation using 12%

trichloroacetic acid (Rowland, 1938). Following the work of Rowland (1938), essentially all of the protein and non-protein nitrogen fractions in milk had been characterized. The properties of milk and the products created from it are largely affected by the protein in the milk. Protein accounts for about 3.3% of the total composition of bovine milk (Walstra et al., 2006) (Table 1.1).

The major proteins in milk can broadly be placed into two categories: caseins and serum proteins. The caseins are defined by the protein which precipitates from milk upon acidification to pH 4.6 at 20 °C. The proteins remaining are considered serum or whey proteins. Casein accounts for about 80% of the protein in milk, while serum proteins contribute the remaining 20% (O'Mahony and Fox, 2013, 2014; Walstra et al., 2006). Along with protein, many other components of milk are used as ingredients in food today including the lipid portion, carbohydrates such as lactose and galacto-oligosaccharides, and minerals such as whey permeate (Beucler et al., 2006; de Boer, 2014; Geilman et al., 1992; Lucey and Horne, 2009; McSweeney and Fox, 2009; Paterson, 2009; Playne and Crittenden, 2009). The diverse functionalities of dairy proteins like emulsification, gelation, foaming, water binding, or heat stability can drastically impact various characteristics of finished products.

## 2.1. Casein

The term casein does not describe a single protein. Casein is more accurately described as the colloidal mixture of four major proteins,  $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ - and  $\kappa$ -casein, which precipitate when milk is acidified to a pH of 4.6 (O'Mahony and Fox, 2013; Walstra et al., 2006). Caseins exist in large colloidal states known as micelles. The micelles are heat stable, able to withstand heating at 140 °C for 15-20 min at natural milk pH without coagulation (Huppertz, 2016; O'Mahony and Fox, 2014). They have a diameter of approximately 130-160 nm although they can have a much

larger range (O'Mahony and Fox, 2013). Casein micelles are fairly hydrated in their native state as they can bind about 3.7 grams of water per gram of protein. The micelles are able to hold large amounts of calcium and phosphorus as particles called Colloidal Calcium Phosphate or Micellar Calcium Phosphate. Casein allows for a higher level of calcium phosphate to be solubilized in milk than would normally be possible. It has been theorized that along with solubilizing minerals for nutritional reasons, casein micelles stabilize otherwise insoluble calcium phosphate preventing precipitation in the ducts of the mammary gland (Fox & Brodtkorb, 2008; McMahon & Oommen, 2013; O'Mahony & Fox, 2013). All of the caseins,  $\alpha_{S1-}$ ,  $\alpha_{S2-}$ ,  $\beta$ - and  $\kappa$ -casein, have multiple genetic variants as well as post translational modifications such as phosphorylation, glycosylation, or disulfide bonding (Holland and Boland, 2014; Swaisgood, 2003).

In milk, the majority of the casein exists in large colloidal aggregates called micelles. While the existence of micelles consisting of various types of casein molecules has been known for some time now, the exact structure has been debated and various theories have been proposed and refined in recent years. Early models suggested that the larger micelle consisted of smaller sub-micelles which were linked via colloidal calcium phosphate (Huppertz et al., 2018; McMahon and McManus, 1998; O'Mahony and Fox, 2013). This model was initially thought to have been supported by various electron microscopy techniques. It has since been suggested that many of the observations from early electron microscopy were due to artifacts produced by the various techniques used to prepare samples for electron microscopy (McMahon and McManus, 1998). An alternate model has suggested a supramolecular structure where the caseins exist as an interlocked lattice formed via multiple simultaneous interactions (McMahon and Oommen, 2008, 2013). The supramolecular model suggests that calcium bridges between caseins, hydrophobic



interactions between caseins, and polymerization between individual caseins all contribute to the structure of the micelle. A slight variation suggests that nonspherical and incompressible regions within the micelle are formed by self-association of caseins and these regions form a network via links to calcium phosphate nanoclusters (Huppertz et al., 2017, 2018). Many of the differences in structural models focus on the interior of the micelle, as it is generally accepted that the surface of the micelle consists mainly of  $\kappa$ -casein oriented in a way that the hydrophilic tails form a brush-like exterior (O'Mahony and Fox, 2013). The protrusions sterically stabilize the micelle against aggregation with other micelles (Huppertz et al., 2018).

## 2.2. Casein ingredient manufacture

Many of the techniques used to create casein based dairy ingredients take advantage of the physicochemical properties of the caseins and the micelle in order to separate the caseins from the serum (whey) proteins. Traditionally, caseins were only able to be separated via macro-scale filtration after the casein micelle was destabilized to the point where it was no longer soluble (Carr and Golding, 2016). Broadly speaking, there are two methods utilized to produce insoluble casein aggregates, rennet-based and acid-based casein precipitation. Both methods utilize the fact that micelle stability is most affected by the outer layer of  $\kappa$ -casein. Rennet is a broad term for a group of proteolytic enzymes that cleave  $\kappa$ -casein at Phe<sub>105</sub>-Met<sub>106</sub> (Fox et al., 2015a, 2015b). Once the portion of the hydrophilic tail is cleaved, aggregation is caused by charge neutralization and the formation of divalent cation bridges. Acid casein is produced by either direct addition of food grade acidulants, or production of organic acids from bacterial fermentation to decrease the pH to the isoelectric point of casein. Both methods decrease the pH and results in the charge neutralization of  $\kappa$ -casein. The neutral charge on  $\kappa$ -casein causes the collapse of the outer  $\kappa$ -casein layer and prevents any electrostatic or steric repulsion thereby

allowing for micelle aggregation (Carr and Golding, 2016; Holt and Horne, 1996; Horne, 2014). Insoluble caseins are important to the manufacture of cheeses, however, are of little use as an ingredient. A method to produce a soluble casein product from insoluble, acid precipitated casein, has existed since 1900 via neutralization with alkali (U.S. Patent No. 664,318, 1900). Alkali neutralization is still widely used today to produce caseinates. The caseinates are the salt of caseins which were previously precipitated by acid addition. Forming the salt by neutralization allows the caseins to be water soluble again. The properties of caseinates can be affected by the alkali used to neutralize the acid casein. Monovalent alkali such as NaOH or KOH produce caseinates which are highly hydrated caseinates where casein-casein interactions are controlled by electrostatic repulsion (Carr et al., 2002). Divalent caseinates, such as calcium caseinate, form colloidal dispersions of poorly hydrated, compact particles. The functional properties of a caseinate ingredient can be modified by adjusting the ratio of monovalent and divalent caseinates. The use of caseinates, especially sodium caseinate, in RTD protein beverages has mainly been due to their high heat stability, with aqueous solutions remaining soluble following heating at 140 °C for several hours (Carr and Golding, 2016; O'Mahony and Fox, 2013). Along with heat stability, caseinates are also used functionally for their emulsification properties in other various foods (Harper, 2014; Singh and Ye, 2014). Caseinates are generally selected as an ingredient solely on their functional properties as they can be fairly expensive and their sensory attributes are characterized by a host of intense and unpleasant descriptors including cardboard, animal, tortilla/corn chip flavors and bitter taste (Carr and Golding, 2016; Drake et al., 2003, 2008; Smith et al., 2016).

Separation and concentration of milk proteins based on their size by membrane filtration has been used in the dairy industry since the 1980s (Novak, 1992). Membrane filtration has been

used more widely for separation and concentration of whey proteins although it is quickly being adopted to separate caseins as well (Brans et al., 2004; Saxena et al., 2009). Membrane operations are usually described as discrete categories with specific pore sizes indicated by the membrane manufacturer (Table 1.2).

Microfiltration retains the least amount of protein as the pore size is large enough to allow essentially all of the protein except for casein micelles to pass through. Dairy ingredients are manufactured from the separation of skim milk using MF membranes with pore sizes of approximately 0.05 and 0.2  $\mu\text{m}$  (Brans et al., 2004). The use of ceramic membranes is preferred for this application as they have a very narrow range of pore sizes allowing for greater selectivity of micellar casein over serum proteins and thus allow separation of these two protein fractions in discrete streams (Amelia and Barbano, 2013; Nelson and Barbano, 2005). To isolate milk proteins in their native ratio of 80% casein and 20% serum protein, UF can be used (Carr and Golding, 2016). Ingredients which result in a final ratio of casein to serum protein that is unchanged from the native milk 80:20 ratio are either called Milk Protein Concentrate (MPC) or Milk Protein Isolate (MPI) depending on the protein content of the final product (Table 3). The MPC nomenclature is also usually followed by the percent protein, for example MPC 80 contains a minimum 79.5% protein. In addition to MPC and MPI, membrane filtered casein products which have a casein to serum protein ratio adjusted to between 82:18 and 95:5 using microfiltration are called either Microfiltered Milk Protein (MMP), Micellar Casein (MC), or Micellar Casein Concentrate (MCC) (Table 4). It should be noted that although the U.S. Dairy Export Council (USDEC) and American Dairy Products Institute provide standard compositions of these concentrated milk protein products, there is no standard of identity set by the Food and Drug Administration (FDA). The FDA has concluded that it had no questions for USDEC and

ADPI when they expressed their view that these products are Generally Recognized as Safe (GRAS) for the uses described in the GRAS proposal (GRAS Notice Inventory - Agency Response Letter GRAS Notice No. GRN 000504).

### 2.3. Casein proteins

#### 2.3.1. $\alpha_{s1}$ – Casein

$\alpha_{s1}$ -CN comprises about 40% of the casein fraction in milk. The reference protein is  $\alpha_{s1}$ -CN B-8P which has eight phosphorylated serine residues (Ser45, Ser47, Ser64, Ser66, Ser67, Ser68, Ser75, and Ser115) while the 9P variant has an additional phosphorylation at Ser41 (Farrell et al., 2004; Manson et al., 1977). It contains 199 amino acid residues and has a molecular weight of ~23.0 kDa prior to phosphorylation and ~23.6 kDa after phosphorylation (de Kruif and Holt, 2003). Phosphorylation of serine residues is crucial to the stability of the micelle structure and the stabilization of large amounts of calcium (de Kruif and Holt, 2003; Swaisgood, 2003).  $\alpha_{s1}$ -CN is able to form various multimers and the equilibrium between various forms is highly influenced by pH and ionic strength (Huppertz et al., 2018; Schmidt, 1970). As with many of the caseins,  $\alpha_{s1}$ -CN is calcium sensitive, meaning that it can precipitated by the addition of  $\text{CaCl}_2$  (Dalglish and Parker, 1980). It becomes soluble again at higher concentrations (>100 mM) of  $\text{CaCl}_2$ , known as salting-in (Farrell et al., 1988). The binding of calcium to the anionic domain affects the hydrophobicity of other parts of the protein and alters the association characteristics with itself and other proteins (Holland and Boland, 2014; Singh et al., 1989b, 1989a).

### 2.3.2. $\alpha_{S2}$ – Casein

About 10% of the casein fraction is the calcium sensitive phosphoprotein  $\alpha_{S2}$ -CN. The reference protein for  $\alpha_{S2}$ -CN family is  $\alpha_{S2}$ -CN A-11P which contains 207 amino acid residues and has a molecular mass of ~24.3 kDa and a posttranslational mass of 25.2 kDa (Farrell et al., 2004; Huppertz, 2013). In bovine milk, the various forms contain varying amounts of phosphorylation and usually contain between 10 and 13 phosphorylated serine residues (Farrell et al., 2004; Swaisgood, 2003). An important point of difference between  $\alpha_{S2}$ -CN and  $\alpha_{S1}$ -CN is the presence of two cysteine residues in  $\alpha_{S2}$ -CN which can participate in intra- or intermolecular disulfide bonds (de Kruif and Holt, 2003; Rasmussen et al., 1994; Swaisgood, 2003). Intramolecular disulfide bonding occurs as a bond between Cys36 and Cys40, which leads to the predominant (>85%), monomeric, form of  $\alpha_{S2}$ -CN (Rasmussen et al., 1994). Dimers can also form, however the formation does not appear to be of any importance to structural properties or association with other caseins (Rasmussen et al., 1992). Due to the presence of 3 groups of anionic residues it is regarded as the most hydrophilic of the caseins (Farrell et al., 2004; Swaisgood, 2003).  $\alpha_{S2}$ -CN is more sensitive to calcium induced precipitation than  $\alpha_{S1}$ -CN due to the anionic clusters and its hydrophilicity (Toma and Nakai, 1973).

### 2.3.3. $\beta$ – Casein

$\beta$ -casein accounts for about 35% of the casein in milk and it contains 5 serine residues which are phosphorylated (Huppertz, 2013). The reference protein is  $\beta$ -CN A<sup>2</sup>-5P and contains 209 residues with a molecular mass of ~23.6 kDa before phosphorylation and ~24.0 kDa following posttranslational modification (Farrell et al., 2004; Swaisgood, 2003). It is devoid of any cysteine residues which prevents disulfide bonding.  $\beta$ -casein is amphiphilic due to the fact that the *N*-terminus contains many charged residues and is fairly hydrophilic, while the C-

terminus contains very little charge and consists mainly of nonpolar residues resulting in high hydrophobicity (Huppertz et al., 2018; Swaisgood, 2003).  $\beta$ -CN was originally separated from  $\alpha$ -CN and another fraction via zone electrophoresis and alcohol solubility (Hipp et al., 1952; Osborne and Wakeman, 1918; Wake and Baldwin, 1961). Both  $\alpha$  and  $\beta$ -CN are sensitive to calcium, meaning they can precipitate upon the addition of  $\text{CaCl}_2$  (Fox and Brodkorb, 2008; Hipp et al., 1952).  $\beta$ -casein is able to self-associate, at lower temperatures ( $<5^\circ\text{C}$ ) the monomer is the main form, however at temperatures higher than that it can form micelles with itself, or associate with other caseins (Huppertz, 2016; Huppertz et al., 2018; Liu et al., 2013; Swaisgood, 2003). In the native casein micelle,  $\beta$ -CN is able to be reversibly released from the micelle due to the effect of temperature on hydrophobic interactions (Creamer et al., 2004; Downey and Murphy, 1970; Liu et al., 2013; Walstra, 1990). The temperature dependent release of  $\beta$ -CN can result in about 30% of the  $\beta$ -CN being released from the micelle, however the stability of the micelle is not negatively impacted by the release (Davies and Law, 1983; Rose, 1968).  $\beta$ -CN that is cross-linked by colloidal calcium phosphate is associated with other caseins by more than just hydrophobic interaction and is not susceptible to temperature dependent release (Aoki et al., 1990). The selective release of  $\beta$ -CN from the micelle can allow for some modification of the composition during processing and also allow for extraction of  $\beta$ -CN from a mixture of caseins (Downey and Murphy, 1970; Huppertz et al., 2018; Murphy and Fox, 1991; Post et al., 2012).

#### 2.3.4. $\kappa$ – Casein

$\kappa$ -casein composes about 10% of the casein and is the only casein which displays various levels of glycosylation and is considered calcium insensitive unlike the other caseins (Fox and Brodkorb, 2008; Huppertz, 2013; Huppertz et al., 2018; Ono et al., 1980). The reference protein for the family is  $\kappa$ -CN A-1P which has 169 residues and a molecular mass of  $\sim 19.0$  kDa however

the actual mass increases depending on phosphorylation and glycosylation (Farrell et al., 2004; Huppertz, 2013). There are two cysteine residues which allow for the formation of a variety of oligomers via intermolecular disulfide bonds. The disulfide bonds present in  $\kappa$ -casein can participate in heat induced disulfide exchange with other proteins such as  $\beta$ -lactoglobulin and can help to stabilize  $\beta$ -Lg (Holland and Boland, 2014; Huppertz et al., 2018).  $\kappa$ -casein contains two domains, a hydrophobic N-terminus which is capable of interacting with other caseins to help form the micelle, and a more hydrophilic net negative section at the C-terminus (Farrell et al., 2003; Holt and Sawyer, 1993). It is well accepted that  $\kappa$ -CN protrudes from the micelle often described as a “hairy” layer, this layer provides steric hindrance from micelles from coalescing (de Kruif and Zhulina, 1996; Farrell et al., 2013; Holt and Horne, 1996; Horne, 2006; Walstra, 1990). The Phe105-Met106 bond of  $\kappa$ -CN is susceptible to hydrolysis by chymosin and produces *para*- $\kappa$ -CN and glycomacropeptide (**GMP**). This is used in the rennet based cheese making process as the removal of GMP allows for the casein micelles, no longer sterically hindered, to interact and coagulate (Farrell et al., 2013; Huppertz, 2013).

#### 2.4. Whey or serum proteins

Serum proteins comprise about 20% of the protein in bovine milk and are generally defined as the proteins that remain soluble at pH 4.6 after the acid precipitation of casein (Fox, 2008). The serum proteins are commonly referred to as whey proteins as they are generally recovered from whey remaining after removing casein.

Whey proteins and serum proteins are essentially interchangeable. The major factor which differentiates these two categories is the source of the protein. Whey protein refers to if the protein is extracted from rennet coagulated casein or acid coagulated casein while serum protein is the scientific name if the protein is directly extracted from milk via filtration of milk.

The American Dairy Products Institute has standardized the name that serum protein ingredients be called as long as they meet the product definition they can be called “milk whey protein,” “native whey protein,” “milked derived whey protein,” or “milk soluble protein” (American Dairy Products Institute, 2015). Acid whey has become much more prevalent due to the manufacture of Greek yogurt, which has grown from 2% of the total volume of yogurt sales in 2009 to about 23% in 2012 (Boynton & Novakovic, 2013). However, acid whey from Greek yogurt does not have sufficient amounts of protein for concentration, however, acid whey from soft cheeses (cottage cheese) does have sufficient protein to justify concentration as an ingredient (Wherry et al., 2019). As previously mentioned, acid whey has a higher mineral content, while whey produced from rennet coagulation, sweet whey, contains glycomacropeptide (GMP) produced from proteolysis of  $\kappa$ -casein.

## 2.5. Whey ingredient manufacture

There are two main sources of whey, cheese manufacture and caseinate manufacture. Caseinate production utilizes acid precipitation of casein from milk while cheese production coagulates casein following enzymatic cleavage of  $\kappa$ -casein. Whey produced by direct acidification or conversion of a significant portion of lactose into lactic acid is known as acid whey (21CFR184.1979). Whey produced from a procedure without a significant conversion of lactose to lactic acid is known as sweet whey. The vast majority of whey produced is of the sweet whey variety (Kilara and Vaghela, 2018). Although the composition of sweet whey and acid whey is similar there are a few key differences. First, acid whey tends to have a higher amount of minerals, specifically calcium as direct acidification allows for more of colloidal calcium phosphate to be solubilized from the casein micelle (Bansal and Bhandari, 2016). Another large difference is the presence of glycomacropeptide, which is produced from the



enzymatic cleavage of  $\kappa$ -casein as previously mentioned (Lucey, 2014). The remaining proteins in the milk serum and whey are similar.

## 2.6. Whey proteins

### 2.6.1. $\beta$ -Lactoglobulin

$\beta$ -lactoglobulin is the predominant serum protein in bovine milk, accounting for about 58% of the protein in the serum phase (Bansal and Bhandari, 2016). There are a number of genetic variants which have been discovered, however the two most common are  $\beta$ -LG A and  $\beta$ -LG B, the latter being the reference protein for the class (Farrell et al., 2004). There are two disulfide bonds within  $\beta$ -lactoglobulin and one free sulfhydryl group on a cysteine residue all of which make it susceptible to disulfide exchanges aggregation (Kilara and Vaghela, 2018). At pH above 8.0 and below 3.0,  $\beta$ -lactoglobulin exists as a monomer, while it can form an octamer between pH 3.1 and pH 5.1 it only does so at high protein content and low temperature, however at the natural pH of milk,  $\beta$ -lactoglobulin forms a dimer (Sawyer, 2013). Denaturation which is highly dependent on a number of factors is generally considered to occur between 71-82 °C (de Wit and Klarenbeek, 1984; de Wit and Swinkels, 1980). The aggregation is a complex process which has been well studied and is affected by many factors in a protein beverage system such as pH, total protein concentration, protein composition, rate of heating, ionic strength and many other factors (Dalglish, 1990; de Wit and Klarenbeek, 1984; Loveday, 2016; Sawyer, 2013).

### 2.6.2. $\alpha$ -Lactalbumin

The other major protein in the serum phase is  $\alpha$ -lactalbumin which makes up about 20% of the serum phase (Brew, 2013; O'Mahony and Fox, 2013). It contains 4 intramolecular disulfide bonds and no phosphorylations (Brew, 2013). A calcium ion can bond strongly in a

pocket of  $\alpha$ -lactalbumin which is made up of 4 aspartic acid residues.  $\alpha$ -lactalbumin readily renatures following denaturation (O'Mahony and Fox, 2014). So although the protein begins unfolding it does not aggregate as readily as other proteins resulting in its ability to renature following heating. The denaturation temperature is about 61 °C however it may drop much lower at low pH (Brew, 2013; Brodkorb et al., 2016).

### 2.6.3. Glycomacropeptide

Glycomacropeptide is produced by selective cleavage of  $\kappa$ -CN during the rennet coagulation of cheese. The structure contains variable amounts of glycosylation and is very heat stable (Doulton et al., 2003; Holland and Boland, 2014). It is not present to any appreciable extent in whey protein isolates which have been fractionated by ion exchange systems. The main attraction of this fraction is that its lack of phenylalanine residues makes it a good source of protein for people with phenylketonuria (PKU).

### 2.6.4. Other proteins

There are many other proteins that have been described in milk and many have specific functions for health. An example are the immunoglobulins a class of proteins which confer critical parts of the immune system to the neonate but serve little functional properties when it comes to beverage applications (Kilara and Vaghela, 2018). Other examples include bovine serum albumin, lactoperoxidase, lactoferrin, proteose peptone and many others. These fractions may be useful in nutraceuticals however they are not present in amounts high enough to influence the physicochemical properties in protein beverages (Corbo et al., 2014; Farrell et al., 2004; McGregor and Poppitt, 2014; O'Mahony and Fox, 2014).

### 3. PROTEIN STABILITY

#### 3.1. Thermal stability

With regards to individual proteins, thermal stability refers to the protein's ability to retain their native conformations and avoid aggregation (Anema, 2014). The two steps of denaturing and then aggregation are considered two separate steps however, while denaturation can be a reversible process where a protein may fold back into its native state upon cooling, aggregation is for most situations irreversible. When evaluating the denaturation of proteins, an important technique used is differential scanning calorimetry (DSC) which evaluates the change in heat capacity of the sample over a range of temperatures (Privalov, 1974). DSC can detect the amount of additional energy needed to overcome the Gibbs free energy and results in the production of an endothermic transition peak (de Wit and Swinkels, 1980; Tanford et al., 1959). Many factors such as pH, protein concentration, protein purity, heating rate, and ionic strength can affect the temperature of denaturation as well as secondary aggregation processes (de Wit and Klarenbeek, 1984).

#### 3.2. Electrostatic stability

The stability of proteins based on electrostatic interaction and aggregation is a well studied and understood concept. The charge of a protein is determined by the composition of amino acids which make up the protein as well as posttranslational modifications such as phosphorylation of side chains, and finally the pH of the solution that the protein is in.

The isoelectric point (**pI**) of a specific protein is the pH where the net charge that specific protein is zero. If there aren't like charges to cause individual proteins to repulse each other they are more likely to aggregate with due to other influences such as hydrophobic interactions, van der waals forces (de Kruif and Holt, 2003; Huppertz et al., 2018). The casein micelle as

previously mentioned is stabilized by the  $\kappa$ -CN brush on the outside. The brush provides steric stabilization but is also highly charged and provides electrostatic repulsion to the micelle as well. As the pH is reduced, there are two main consequences, increased solubility of the calcium phosphate clusters which bridge caseins, and decreased electrostatic and steric repulsion of the casein micelles as a whole (McMahon and Oommen, 2013). Whey proteins show greater stability at reduced pH values, that is pH values that are further away from their isoelectric point (5.35 for  $\beta$ -lg and 4.80 for  $\alpha$ -la) (Edwards and Jameson, 2014).

## 4. READY TO DRINK PROTEIN BEVERAGES

### 4.1. Stability

In the context of beverages, stability refers to the ability of the beverage to maintain a homogeneously dispersed phase of particulates within a fluid continuous phase. There are two main classes of instability which occur within protein beverages, initial stability and shelf life stability. The initial stability is commonly referred to as thermal stability meaning the beverage remains stable, as defined above, following the thermal treatment of the beverages. It should also be noted that the initial stability can be affected by a number of factors such as pH, ionic strength, the conditions of thermal processing, the initial protein concentration and other factors described later in this section (de Wit, 1990; Eshpari et al., 2014; Vardhanabhuti and Foegeding, 2008; Walstra et al., 2006).

The shelf stability of beverages is largely determined by phase separation over time or by gel formation. The phase separation is fairly well understood and is driven by Stoke's law where particle density and continuous phase viscosity are the main factor which determine the rate of separation. Secondary aggregation of proteins can affect the values involved in Stoke's law, however the loss of stability would still be modeled by this law. Gel formation is much less

understood but is thought to be mainly driven by protein hydrolysis by heat stable plasmins over time (Anema, 2019; Gaucher et al., 2008; Kelly and Foley, 1997; Metwalli et al., 1998; Rauh et al., 2014). Another possible explanation of age gelation could be the formation of a cross-linked protein network from  $\kappa$ -CN depleted casein micelles (Anema, 2017, 2019).

#### 4.1.1. pH

Ready to Drink beverages are divided into two main categories, neutral pH beverages and acidified beverages. Neutral beverages have a pH between 4.6 and 7.5 and acidified beverages ideally have a pH of between 2.5 and 3.0 (Etzel, 2004; Rittmanic and Burrington, 2006). At low pH, the electrostatic destabilization of casein micelles, and the solubilization of colloidal calcium phosphate bridges results in the subsequent precipitation of casein micelles. This feature of the casein prevents any MPC or MCC from being used in low pH protein drinks. However, when only whey proteins are used in the formulation the thermal stability of the beverages appears to be enhanced by reducing pH further from the isoelectric points of the whey proteins. An optimum pH in whey protein beverage systems of about 3.5. The whey proteins are far enough away from their isoelectric point where they are heat stable and electrostatically stabilized and form a clear solution, while at pH 4.0 the beverages are turbid which shows larger protein aggregates have formed (Etzel, 2004; LaClair and Etzel, 2010). A further reduction in pH would likely only reduce sensory acceptance of the beverages and also lead to the beginning of acid induced protein hydrolysis. The reduced pH is also able to prevent the formation of disulfide bonds which also increases the heat stability of the whey protein solutions (Harwalkar, 1980; Shimada and Cheftel, 1989).

It should be noted that proteins isolated from plant sources are a growing trend in the RTD beverage category. The common sources are soy, pea, and oat protein, but many other

sources are available. One of the largest issues with using plant proteins is they are associated with various off flavors caused compounds extracted with the protein or formed during the extraction process. The amount of flavor is heavily dependent on the processing of the protein (US 7,037,547 B2, 2006; Lei and Boatright, 2007; Paulsen, 2009). Many of the off flavors include green, cardboard, beany, nutty, and cereal along with a variety of others (Chambers et al., 2006). Another problem associated with plant based proteins is they tend to be more prone to gritty textures and astringency which negatively impact the overall mouthfeel of the beverage (Paulsen, 2009). The extent of these negative attributes can vary by the specific plant source as well as the individual manufacturers of the isolated protein and beverages.

#### 4.1.2. Thermal process

The main difference between retort and aseptic processing is that for retort processing the product is put into a container and processed in its final container while aseptic processing sterilizes the product and container separately and fills within the confines of a sterile environment (Bylund, 1995). Given the pH and thermal treatments, the specific protein(s) used in these beverages is also limited based on the physico-chemical limitations. In acidic beverages, caseins cannot be used as they precipitate at acidic pH (4.6) as discussed above, while whey proteins are soluble at low pH and are able to produce a beverage with low turbidity. At neutral pH, the major whey proteins unfold at various temperatures in the range of 62 to 78 °C which can lead to aggregate formation (Anema, 2014; Brodkorb et al., 2016; de Wit, 1990, 2009). Processing at the temperatures required for a neutral pH beverage to achieve commercial sterility would cause whey proteins to destabilize and either precipitate or form a gel. Caseins, however, are very heat stable with some casein ingredients such as sodium caseinate being able to withstand heating at 140 °C for hours at pH 7 without visible changes (O'Mahony and Fox,

2014). The rate of heating and the highest temperature are both important factors when processing protein beverages. In general longer periods of time will lead to more denaturation and higher processing temperatures will reduce the stability of protein beverages.

#### 4.1.3. Protein concentration

Consumer desires for more protein has led manufacturers to increase the concentration of protein in a beverage. It has been known that an increase in protein concentration increases the possibility of proteins interacting once denaturation occurs (Anema and Li, 2003; Loveday, 2016; Vardhanabhuti and Foegeding, 2008). It is obvious that increasing the concentration of protein reduces the heat stability of the proteins, therefore it is important to select proteins which can act synergistically such as the attachment of  $\beta$ -LG with the surface of casein micelles to stabilize the system as a whole while adding protein to the beverage (Dalglish, 1990; Donato and Guyomarc'h, 2009; Foegeding et al., 2002; Wagoner and Foegeding, 2017; Wagoner et al., 2016).

#### 4.1.4. Salts and ionic strength

Ionic species are commonly present in milk systems and exist in an equilibrium between their ionic species and their colloidal state. One of the most influential minerals is the colloidal calcium phosphate within the casein micelle. The reduction of pH causes the calcium phosphate in the increase in solubility which can result in destabilization of the micelle (Anema, 2009). Calcium is an especially important ionic species as it is divalent and able to bridge connections between proteins. Various chelators such as citrate, or polyphosphates are used to bind free calcium in protein beverages which can increase the heat stability of the beverages by reducing the amount of free calcium available for aggregation (Carr et al., 2002; Lucey and Horne, 2009; Renhe et al., 2018). Other than calcium, NaCl has also been investigated for its ability to modify

the heat stability of proteins in solution. The cation is able to screen the charged groups in a protein and will reduce the charge repulsion leading to greater aggregation (Vardhanabhuti and Foegeding, 2008).

#### 4.2. Flavor binding

Flavor perception in protein beverages is an important consideration when formulating due to the importance of flavor in contributing to the overall consumer acceptance of the product. It has been well documented that protein in a food matrix can have a large impact on the amount of flavor perceived in the final product (Damodaran and Kinsella, 1980b, 1980a; Hansen and Heinis, 1991; Kühn et al., 2006; Kühn et al., 2008; O'Neill, 1996). Whey proteins are known to bind many flavor compounds more strongly than caseins (Hansen and Heinis, 1991; McNeill and Schmidt, 1993; O'Neill, 1996). Vanillin in particular is bound to whey proteins and the perception of vanilla flavor is reduced in whey protein containing beverages (Hansen and Heinis, 1991).

#### 4.3. Flavors from proteins

Proteins have many sensory contributions more than simply the binding of added flavorings. Both spray dried milk and whey proteins have distinct flavors which can carry through into protein beverages (Carter and Drake, 2018; Oltman et al., 2015; Smith et al., 2016). Even prior to heat treatment, protein type and amount can affect the astringency of the product (Beecher et al., 2008). The whey proteins, specifically  $\beta$ -LG are capable of increasing the astringency of beverages, especially acidic beverages at a pH of about 3.4 and high protein concentrations (Beecher et al., 2008; Vardhanabhuti et al., 2010). Although less of a factor at neutral pH, astringency from proteins, specifically whey proteins, should be taken into account (Andrewes et al., 2011).



Whey proteins also directly contribute to the flavor of the final beverage when neutral pH beverages are processed at high temperatures. The high sulfur content and free sulfhydryl groups present in denatured whey proteins are able to react and create a variety of flavor active sulfur compounds (Creamer et al., 2004; Smith et al., 2016). These sulfur compounds lead to flavors often described as cooked or eggy (Jo et al., 2018). Caseins have a much lower sulfur content and therefore do not contribute much to the sulfurous flavors created from the heat treatment of the beverages (Farrell et al., 2004).

Although not focused on in this review plant based proteins are becoming more popular in the ready to drink category and present all of the challenges of protein instability discussed above to varying extents based on the source as well as the purity of the protein (Nishinari et al., 2014, 2018). Common plant sources include soy, pea and rice (Corbo et al., 2014). The largest challenge associated with plant proteins is that they do not have a consistent protein composition and have many different individual proteins depending on the extraction technique used which may also co-extract or form many flavor-active compounds commonly associated with these proteins (Aryee et al., 2018; Nishinari et al., 2014). The inconsistent nature of plant protein extracts and their distinct flavor contributions makes their utilization for ready to drink protein beverages a challenge.

## 5. CONCLUSIONS

As evident by the nature of the initial discovery and classification of casein, dairy ingredients that have casein are not soluble at pH of 4.6 which prevents the use of MPC and MCC in any acidified beverage. In contrast, whey protein is soluble over a much larger range, including acidic environments. Whey proteins are least soluble and subsequently least heat stable in the pH range of 4.8 to 5.2 as this is the range of isoelectric point of the major whey proteins

(Kilara and Vaghela, 2018). At  $\text{pH} < 4.8$  and  $\text{pH} > 5.2$  the solubility and heat stability of whey proteins increase which makes whey protein ideal for use in acidified beverages. The objectives of this thesis are to evaluate and explore the concentration of protein as well as varying ratios of casein and whey proteins in a model neutral pH RTD protein beverage system. Both sensory and instrumental measures of the beverages over 8 weeks of storage were evaluated. The results of this study will be important first steps in evaluating a neutral pH, high protein beverages and provide a direction for future research to take.

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Table 1.1. Average composition of raw bovine milk (adapted from Walstra, Wouters, & Geurts, 2006).

Component	Average Content in Milk (% w/w)	Average Content in Dry Matter (% w/w)
Water	87.1	—
Lactose	4.6	36.0
Fat	4.0	31.3
Protein	3.3	25.8
Casein proteins	2.6	20.4
Serum proteins	0.7	5.5
Minerals	0.7	5.5
Organic Acids	0.2	1.3

Table 1.2. Common pore size of membrane filtration categories.

Technology	Pore Size
Microfiltration (MF)	0.1 – 10 µm
Ultrafiltration (UF)	0.01 – 0.1 µm
Nanofiltration (NF)	0.001 – 0.01 µm
Reverse Osmosis (RO)	< 0.001 µm

Table 1.3. Standard composition of commercially available MPC and MPI (Taken from American Dairy Products Institute’s Concentrated Milk Proteins Standard).

Product	Min Protein (%)	Max Fat (%)	Max Lactose (%)	Max Ash (%)	Max Moisture (%)
MPC 40	39.5	1.25	52.0	10.0	5.0
MPC 42	41.5	1.25	51.0	10.0	5.0
MPC 56	55.5	1.50	36.0	10.0	5.0
MPC 70	69.5	2.50	20.0	10.0	6.0
MPC 80	79.5	2.50	9.0	8.0	6.0
MPC 85	85.0 <sup>a</sup>	2.50	8.0	8.0	6.0
MPI	89.5 <sup>a</sup>	2.50	5.0	8.0	6.0

<sup>a</sup> Protein content ≥ 85.0 % is reported on a dry basis all other parameters are reported ‘as is.’

Table 1.4. Standard composition of commercially available MMP/MC/MCC (Taken from American Dairy Products Institute's Concentrated Milk Proteins Standard).

Product (MPP/MC/MCC)	Min Protein (%)	Max Fat (%)	Max Lactose (%)	Max Ash (%)	Max Moisture (%)
42	41.5	1.25	51.0	6.0	5.0
70	69.5	2.50	16.0	8.0	6.0
80	79.5	3.00	10.0	8.0	6.0
85	85.0 <sup>a</sup>	3.00	3.0	8.0	6.0
90	89.5 <sup>a</sup>	3.00	1.0	8.0	7.0

<sup>a</sup> Protein content  $\geq$  85.0 % is reported on a dry basis all other parameters are reported 'as is.'

## **CHAPTER 2. PROTEIN AMOUNT AND MILK PROTEIN INGREDIENT EFFECTS ON SENSORY AND PHYSICOCHEMICAL PROPERTIES OF READY-TO-DRINK PROTEIN BEVERAGES**

### **1. ABSTRACT**

This study evaluated the role of protein concentration and milk protein ingredient (serum protein isolate (SPI), micellar casein (MCC), or milk protein concentrate (MPC)) on sensory and physicochemical properties of vanilla ready-to-drink (RTD) protein beverages. The RTD beverages were manufactured from 5 different liquid milk protein blends (100% MCC, 100% MPC, 18:82 SPI:MCC, 50:50 SPI:MCC, and 50:50 SPI:MPC) at 2 different protein concentrations 6.3% and 10.5% protein (15 or 25 g protein per 237 mL) with 0.5% w/w fat and 0.7% w/w lactose. Dipotassium phosphate, carrageenan, cellulose gum, sucralose and vanilla flavor were included. Blended beverages were pre-heated to 65°C and homogenized (20.7 MPa) and cooled to 8°C. The beverages were then preheated to 92°C, and then ultrapasteurized (141°C, 3 sec) followed by vacuum cooling to 92°C and homogenization again (17.2 MPa first stage, 3.5 MPa second stage). Beverages were then cooled, filled into sanitized bottles and stored at 4°C. Initial testing of RTD beverages included proximate analyses and aerobic plate count and coliform count. Volatile sulfur compounds, vanillin, particle size, and sensory properties were evaluated through 8 weeks. Astringency and viscosity were higher and sweet aromatic/vanillin flavor was lower in beverages containing 10.5% protein compared to 6.3% protein ( $P<0.05$ ) and sulfur/eggy flavor, astringency and viscosity were higher and sweet aromatic/vanillin flavor were lower in beverages with higher serum protein as a percentage of true protein within each protein content ( $P<0.05$ ). Volatile compound analysis of headspace vanillin and sulfur compounds was consistent with sensory results, beverages with 50% serum protein as percentage of true protein and 10.5% protein had the highest concentrations of sulfur volatiles and lower vanillin compared to other beverages ( $P<0.05$ ). Sulfur volatiles and sulfur/eggy flavors and vanillin and sweet

aromatic/vanillin flavor decreased in all beverages with storage time ( $P<0.05$ ). Particle size was larger in beverages with higher serum protein as a proportion of true protein and the particle size increased with storage time ( $P<0.05$ ). These results will enable manufacturers to select and or optimize protein blends to better formulate RTD beverages to provide consumers with a protein beverage with high protein content and desired flavor and functional properties.

## 2. INTRODUCTION

Consumers continue to seek out products that offer benefits for their health and wellness. A recent survey indicated that 76% of shoppers ages 18-80 y in the United States made small changes in their diet throughout the year to achieve an overall healthier diet (The International Food Information Council, 2016). In that same survey, protein was the most sought after nutrient with 64% of respondents indicating that they were trying to consume more protein in their diet. The convenience and variety of ready-to-drink (**RTD**) protein beverages attracts new consumers who are seeking ways to increase protein intake. Dairy proteins, specifically milk and whey proteins, are the most desirable types of protein in RTD beverages and  $\geq 15$  g of protein per serving are favored (Will S. Harwood and Drake, 2019; Oltman et al., 2015). RTD beverages currently on the market have a broad range of protein content from 10 to 40 grams of protein per serving. However, the reported variability of the amount of protein per serving can partially be attributed to inconsistent serving sizes which range from 236 to 591 mL per serving. Manufacturers are increasing concentration of protein in beverages in response to consumer demand which presents a technical challenge to formulate and manufacture these products as thermal and shelf stability, as well as flavor of these beverages are impacted by higher concentrations of protein.

There are two main categories of RTD beverages, neutral pH beverages and acidified beverages. Neutral beverages generally have a pH between 4.6 and 7.5 and acidified beverages ideally have a pH of between 2.5 and 3.0 (Etzel, 2004; Rittmanic and Burrington, 2006). The pH range of the beverages dictates the thermal treatment the beverages will require as an acid pH (<4.6) lowers the heat resistance of bacteria (Palop and Martínez, 2005). Generally speaking, acidic beverages are traditionally processed by “hot fill” where the beverage is heated to at least 83°C (target temperature 90–95°C), then filled into a container and inverted while still hot (Frederick et al., 2010; Rushing and Foegeding, 2010).

Shelf-stable neutral pH beverages are required to undergo either retort processing (115–125°C for about 10–40 min), or Ultra High Temperature (**UHT**) treatment (135–150°C with a hold time of 2–6 s) followed by cooling and filling aseptically into previously sterilized containers. The pH and thermal treatments determine the specific protein(s) which can be used in RTD beverages. In acidic beverages, casein ingredients cannot be used as they precipitate at acidic pHs (<4.6), while whey proteins are soluble at low pH. At neutral pH, whey proteins are not heat stable. Caseins, however, are very heat stable with some casein ingredients such as sodium caseinate being able to withstand heating at 140°C for hours at pH 7 without visible changes (O’Mahony and Fox, 2014).

The physicochemical properties of casein and whey protein ingredients influence the desired nutritional profile, functionality and sensory properties of RTD protein beverages. Many studies have investigated heat stability of individual dairy proteins, at various concentrations and matrix effects such as pH, ionic strength and the addition of various complexes (de Wit, 2009; Donato and Guyomarc’h, 2009; Vardhanabhuti and Foegeding, 2008; Yong and Foegeding, 2008, 2010). Recently, Kelleher et al. (2018) compared physical properties of whey protein

isolate solutions with 4, 6, and 8% (w/w) protein and a pH of 6.8 processed by direct or indirect heating at 121°C and 135°C for 2s. However, to our knowledge, previous studies have not evaluated the role of different milk protein blends and protein concentration on sensory and functional properties of RTD protein beverages. The goal of this study was to evaluate the role of protein concentration and milk protein ingredient (serum protein isolate (**SPI**), micellar casein (**MCC**), or milk protein concentrate (**MPC**)) on sensory properties, volatile compound analysis, and particle size analysis of model vanilla flavored RTD protein beverages.

### 3. MATERIALS AND METHODS

#### 3.1. Experimental design

Beverage formulations were based on a factorial arrangement (protein concentration and protein composition) in a complete block design with repeated measures across time. The beverage treatments (n=10 total) (Table 2.1) were combinations of true protein (**TP**) content (6.3 and 10.5% w/w) and 5 compositions of dairy protein ingredients (100% MCC, 100% MPC, 18:82 SPI:MCC, 50:50 MCC:SPI, and 50:50 MPC:SPI) (Table 2.2). The protein concentrations were selected to represent 15 or 25 g protein per 240 mL serving (Will S. Harwood and Drake, 2019). The protein blends represented various milk protein sources available to beverage processors. Proximate analysis, particle size analysis, descriptive sensory analysis, standard plate count and coliform count were performed on the beverages. All analyses, excluding proximate analysis were performed every two weeks, through an 8 week period. The entire experiment was replicated twice.

#### 3.2. Initial stability testing

Small scale testing was done using an oil bath to simulate the thermal process to determine which of the formulations would not form a gel during processing. Each treatment was blended using a stick blender. The blended treatments were preheated to 65°C in a water bath and homogenized at 20.7 MPa (17.2 MPa first stage, 3.5 MPa second stage) using a Panda 2K Type NS10001L2K (GEA Niro Soavi S.p.A, Parma, Italy). After homogenization, the samples were cooled to 8°C. Samples were equilibrated to 20°C and 2 mL of the mixed and homogenized solutions were placed into Pyrex Vista borosilicate test tubes with rubber lined caps (16 mm OD, Corning Incorporated Life Sciences, Oneonta, NY, USA). The Neslab EX7 oil bath (Thermo Scientific, Waltham, MA, USA) was heated to 150°C. The samples were placed inside and

agitated for 62 seconds then held quiescently for 6 seconds. The heating time in the oil bath to achieve a minimum internal temperature of 142°C for the 6 s holding period was determined from a previous experiment by plotting the temperature profile using a MSR 145B8 FT2/160 temperature data logger (MSR Electronics, Seuzach, Switzerland). The samples were removed from the oil bath, cooled in an ice bath at 0°C for 1 min, then visually evaluated for gel formation.

### 3.3. Milk-based ingredients

#### 3.3.1. Micellar casein concentrate

Liquid MCC was made the day before beverage formulation; raw skim milk was obtained from the North Carolina State University Dairy Enterprise System (NCDES) and pasteurized with a high temperature short time (**HTST**) treatment (11.34 kg/min) using a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC) at 72°C with a hold time of 16 s. The pasteurized skim milk was cooled to 4°C, prefiltered by a Nexis T Filter (NXT 10–30U-M7S, Pall Corporation, Port Washington, NY, USA) and stored at 4°C in a 1,200 kg jacketed stainless steel tank (type A & G, Chester-Jensen Company, Chester, PA). The pasteurized and pre-filtered skim milk was heated using a plate heat exchanger (serial no. G201400849, Plate ID: SR1, SPX Flow Technology, Greensboro, NC, USA) to 50°C, weighed, and poured into the microfiltration (**MF**) balance tank (190 kg, NUVAT for cheese making, Meyer-Blank Company, St Louis, MO, USA) jacketed with 50°C water.

A 3-stage, 3× microfiltration (**MF**) process described by Zulewska and Barbano (2014) was used to produce a 95% serum-protein-removed MCC with TP concentration between 12.8 and 13.6% with the following differences in procedure. The cleaning before and after processing, clean water flux, and warm-up procedure was done as described by Zulewska et al. (2009). The



MF was started by filling the flow system with deionized (**DI**) water and the retentate and permeate removal rates were set to 48.0 and 110 L/h, respectively, to achieve a 3× MF mass/mass concentration factor, and cross-flow (**CF**) velocity was kept around 7.0 m/s.

The first 160 kg of milk taken into the MF system were used to flush water out of the system. Retentate and permeate outlets collected during the flush stage were discarded. MF stage 1 began by adding 370 kg more milk at 50°C to the balance tank. For the rest of the filtration both retentate and permeate outlets were collected, weighed, and the CF was controlled based on these weights. The pasteurized and pre-filtered warm skim milk was microfiltered at 50°C with a MF system (Tetra Alcross MFS-7, TetraPak Filtration Systems) equipped with 0.1 µm nominal pore diameter graded permeability ceramic Membralox (model EP1940GL0.1u, AGP1020, alumina, Pall Corporation, Port Washington, NY, USA) membranes (surface area of 1.68 m<sup>2</sup>, membrane length of 1.02 m). The MF retentate and permeate removal rates were set to theoretical target values to achieve a 3× MF mass/mass concentration factor with the retentate removal rate set to 48.0, 54.0, and 52.8 L/h and the permeate removal rate at 110, 120, and 120 L/h for stages 1, 2, and 3, respectively. The retentate recirculation pump rate was increased gradually to maintain a cross-flow velocity of about 7.0 m/s during processing. The retentate removal rate was adjusted during the run to control the concentration factor based on flux, by mass balance, and composition data, determined by mid-infrared (**MIR**) spectrophotometer (Lactoscope FTA, Delta Instruments, Drachten, Netherlands) both values were recorded every 15 minutes.

The second and third stages were diafiltration stages with same weight of DI water added to the retentate as permeate removed in the previous stage. The MF system was run continuously and not stopped between stages as in Zulewska and Barbano (2014). The stage one 3× retentate

plus DI water was heated to 50°C and fed into the MF for stage 2 of the process. At the beginning of stage 2, the stage 1 retentate plus water mixture MF was recycled for 15 min with both the retentate and permeate going back into the feed tank. The protein concentration of MF retentate was checked using the MIR milk analyzer, and once a protein concentration of between 8.4 and 8.6% was achieved, the collection of stage 2 MF retentate and permeate started. The same diafiltration process was done for stage 3, however the permeate removal rate was increased until a retentate protein concentration of 12.8 to 13.6% was achieved. Once target protein was achieved, the retentate was collected directly into sanitized milk cans and cooled to 10°C using a glycol water bath. Following the initial cooling, the cans were stored at 4°C in a walk-in refrigerator overnight.

### 3.3.2. Milk protein concentrate

Raw skim milk was obtained from NCDES. The raw skim milk was processed using the same procedure as for MCC production before feeding to the ultrafiltration (UF) system. A 50°C, 2-stage UF process was used to produce liquid MPC with a protein concentration of about 15% (w/v). Two UF membrane systems (model Pellicon 2, Millipore Inc., Billerica, MA, USA) were set up in parallel with each one of them equipped with 5 polyethersulfone cartridge membrane filters (model P2B010V05, nominal separation cutoff = 10,000 kDa, surface area = 0.5 m<sup>2</sup> per cartridge). A variable-speed peristaltic pump (model 77410-10, Cole-Palmer, Vernon Hills, IL, USA) equipped with 2 model 77601-00 pump heads (Cole-Palmer, Vernon Hills, IL, USA) with silicone tubing (model 96440-73, Cole-Palmer, Vernon Hills, IL, USA) was used. The 1% w/v sodium hydroxide (food grade, Sigma-Aldrich, St. Louis, MO) membrane storage solution in the UF unit was flushed out with 50°C DI water for 20 min. A UF clean water flux was taken by collecting flow from both permeate outlets for 30 s. The UF clean water flux was

1,300 to 1,500 g collected for 30 s at 50°C with no back pressure. During the UF processing, about 124 to 138 kPa back pressure was applied on the UF retentate side of the membranes. The retentate outlets were put into UF balance tank for recirculation in a batch concentration mode for each stage. The UF permeate was collected, weighed, and discarded. The UF retentate and permeate were collected to check flux and composition every 30 min. The stopping point of UF stage 1 was defined as when the UF retentate reached 74% solids (wt/vol) determined by MIR. The second stage of the UF process was a DF stage. The diafiltration water was HTST pasteurized (11.34 kg/min) with a plate heat exchanger (model T4 RGS- 16/2, SPX Flow Technology, Greensboro, NC, USA) at 72°C for 16 s and cooled to 50°C. The DI water added for stage 2 was equal to the amount of permeate removed in UF stage 1. The UF stage 2 was stopped when the protein concentration in the UF retentate reached the target protein content of 14.5 to 15.5% as measured using the MIR milk analyzer. Both retentate and permeate of UF stage 2 were collected and weighed. The liquid MPC (second stage retentate) was collected directly into sanitized milk cans and cooled to 10°C using a glycol water bath. Following the initial cooling, the cans were stored at 4°C in a walk-in refrigerator overnight.

### 3.3.3. Serum protein isolate

Frozen serum protein isolate (SPI) was purchased from South Dakota State University (Brookings, SD, USA) and stored at -20°C. Frozen SPI was thawed at 4°C in a walk-in cooler for 3 days. The SPI consisted of 22.3% protein, 0.15% fat, and 0.52% lactose as determined by the proximate analysis methods listed below.

#### 3.3.4. Cream

High temperature short time pasteurized cream containing about 41% fat (w/v) was obtained from the North Carolina State University Dairy and stored at 4°C prior to use. The composition of the cream was verified by MIR (Lactoscope FTA, Delta Instruments, Drachten, Netherlands) prior to formulation.

#### 3.3.5. Lactose and water

Lactose monohydrate (Hilmar 5120 Refined Edible Lactose 200 mesh, 25 kg) was donated by Hilmar Ingredients (Hilmar Ingredients, Hilmar, CA, USA). Potable water was processed to produce DI water using a deionizing water system (unit number: 1933-2, Mar Cor, Raleigh, NC, USA).

#### 3.3.6. Additional ingredients

All beverages also were formulated to include 0.015% (w/w) carrageenan (Ticaloid 780, TIC Gums, Belcamp, MD, USA), 0.4% (w/w) cellulose gel (Ticaloid Pro HC 988, TIC Gums, Belcamp, MD, USA), 0.03% (w/w) sucralose (100% Sucralose Powder, Sweet Solutions LLC, Plainfield, IL, USA), 0.15% (w/w) dipotassium phosphate (Consolidated Chemical, Quakertown, PA, USA), and 0.5% (w/w) vanilla flavoring (Glanbia Nutritionals, Carlsbad, CA, USA) (Table 2.2).

#### 3.4. Formulation

Formulations were calculated using concentrations of fat (0.5% w/w), TP, casein as a percent of true protein (**CN%TP**), SP, and lactose (0.7% w/w) (Misawa et al., 2016). The additional ingredients which were noted above were constant across all beverage formulations.

Target percent lactose and fat were also constant across beverage formulations. These values were entered into a Microsoft Excel (Redmond, WA) spreadsheet designed to use the Generalized Reduced Gradient (**GRG**) optimization solver function in Excel with the multistart option selected. Desired final target composition (fat, TP, CN%TP, SP, lactose, and final weight (35.0 kg)) of each batch was also entered into the spreadsheet (Figure 2.1). The output of the optimization function for each formulation was saved into the final formula table used for processing.

### 3.5. Processing

Liquid ingredients (DI water, cream, and protein ingredients) were weighed into a sanitized milk can and mixed. Dry ingredients were weighed out and combined. The powder was slowly added to the wet ingredients with constant mixing. The final mix for each beverage was allowed to continue to mix for an additional five min prior to being preheated to 65°C using a Microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC, USA) and homogenized at 20.7 MPa (17.2 MPa first stage, 3.5 MPa second stage) using a 2-stage homogenizer (NS2006H, GEA Niro Soavi, Parma, Italy) followed by cooling to 8°C using the tube-in-tube cooling section of the Microthermics unit, placed into sanitized milk cans and stored at 4°C until all samples were homogenized, the system was reconfigured, and heat sterilized (approximately 3 hours total).

The EVHV was then reconfigured for final heating with DSI (direct steam injection) and filling with an Ultra Clean Fill Hood/Sterile Product Outlet. The crossover time previously determined by flushing water out of the system and analyzing the freezing point (Cryoscope, model no. 4250, Advanced Instruments Inc., Norwood, MA, USA) and the composition (MIR analysis) of the outlet was used to determine the amount of time a sample was processed before

collecting in order to prevent crossover between samples. This crossover time was the minimum time between the start of sample introduction and the collection of the final product.

The entire system was heat sterilized prior to processing by heating until the product outlet reached at least 121°C and held for at least 30 min. Following heat sterilization, the beverages were introduced into the system and preheated to 92°C. Beverages were heated to a final temperature of 141°C using direct culinary steam injection (model LG-30, Electro-Steam Generator Corp., Alexandria, VA) via a Microthermics Steam Injection Module (Microthermics, Raleigh, NC, USA). The beverages were held for 3 seconds at 141°C, then were vacuum cooled to 92°C and homogenized again at 20.7 MPa (17.2 MPa first stage, 3.5 MPa second stage). The beverages were cooled to 8°C using the tube-in-tube cooling section of the Microthermics unit, then filled into sanitized bottles (473 mL (16 oz), Item ID B252SS, Container and Packaging Supply Inc, Eagle, ID, USA) using the clean fill hood. Samples were stored at 4°C until testing.

### 3.6. Analysis methods

#### 3.6.1. Proximate analysis

Milk-based ingredients were analyzed in triplicate using the following analytical methods: Total Solids (**TS**) by direct forced-air method (AOAC International, 2012; method number 990.20), fat by ether extraction, (AOAC International, 2012; method number 989.05), lactose by spectrophotometric enzymatic method (AOAC International, 2012; method number 2006.06), total nitrogen (**TN**) (AOAC International, 2012; method number 991.20), non-protein nitrogen (**NPN**) (AOAC International, 2012; method number 991.21), and non-casein nitrogen (**NCN**) (AOAC International, 2012; method number 998.05). True protein was calculated as TN minus NPN multiplied by 6.38, casein was calculated as TN minus NCN multiplied by 6.38, and serum protein content was calculated by subtracting NPN from NCN and multiplying by 6.38.

### 3.6.2. Microbiological testing

Beverages were tested after processing and at every time-point for aerobic plate count (**APC**) and coliform count (AOAC International, 2012; method 989.10) (Petrifilm Aerobic Count Plate and Petrifilm Coliform Count Plate, 3M Food Safety, Saint Paul, MN, USA) to evaluate microbial quality.

### 3.6.3. Descriptive sensory analysis

Sensory analysis was conducted in accordance with the North Carolina State University Institutional Review Board for the Protection of Human Subjects in Research regulations. Sensory properties of the beverages were evaluated by 7 trained panelists at each time point. Each panelist (3 females, 4 males, ages 21 to 55 y) had a minimum of 80 h of previous experience evaluating flavor and texture attributes of dairy products using the Spectrum method (Meilgaard et al., 2007), and at least 40 h of experience with evaluation of fluid milk sensory attributes using an established sensory language (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Attributes evaluated included aroma intensity, sweet aromatic/vanilla, cooked milky, sulfur/eggy, sweet taste, astringency, metallic mouthfeel and viscosity. Beverages (30 mL) were dispensed into 59 mL soufflé cups labeled with random 3-digit blinding codes and sealed with lids (Dart Container Corp., Mason, MI). The beverages were prepped and stored under reduced light to prevent the formation of light oxidized flavors, and were evaluated at 21°C. Each panelist evaluated each treatment at each time point in duplicate.

### 3.6.4. Headspace-SPME-GC-MS of vanillin

Headspace vanillin content was determined using an Agilent 7820 gas chromatograph (**GC**) with 5975 MSD (Agilent Technologies Inc., Santa Clara, CA, USA) and a ZB-5ms column

(30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Torrance, CA, USA).

Sample introduction was accomplished using a CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Analytical conditions for GC-MS were adapted from Vazquez-Landaverde et al. (2005). At each time point, five milliliters of milk along with 20 µL of internal standard (benzyl benzoate in methanol at 200 µg/L) was added to 20 mL solid phase microextraction (SPME) autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA, USA) in triplicate. Vials were equilibrated for 25 min at 35°C with 4 seconds of pulsed 250 rpm agitation. A single 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA) 1 cm fiber was used for all analysis. The SPME fiber was exposed to the beverages for 40 min at a depth of 31 mm. The fiber was retracted and injected at 50 mm in the GC inlet for 5 min. The GC oven was initially held at 35°C for 3 min with a gradual increase of 10°C/min to 150°C and held for 1 min then raised at a rate of 20°C/min to 250°C and maintained for 5 min. The SPME fiber was introduced into the inlet using splitless mode at 250°C (0.75 min valve delay). All analyses were performed at a constant flow rate of 1 mL/min with helium. Scanning parameters were set from 30 to 350 m/z to identify compounds of interest. The MS transfer line was maintained at 250°C with the quadrupole at 150°C and source at 230°C. Vanillin was quantified using the extracted ion of 152 m/z.

### 3.6.5. Headspace-SPME-GC-MS/MS

Selected sulfur compounds (Jo et al., 2018), hydrogen sulfide (H<sub>2</sub>S) and carbon disulfide (CS<sub>2</sub>), were measured using an Agilent 7890B gas chromatograph applied to an Agilent 7000C triple quadrupole mass spectrometer (GC-MS/MS) equipped with a sulfur selective flame photometric detector (FPD) (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a



ZB-5ms column (30 m length x 0.25 mm i.d. x 0.25  $\mu$ m film thickness; Phenomenex, Torrance, CA, USA). Sample introduction was accomplished using a CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Five (5) mL of sample along with 20  $\mu$ L of internal standard (ethyl methyl sulfide in diethyl ether at 1.65 mg/L; Sigma Aldrich, St. Louis, MO, USA) was added to 20 mL SPME autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA, USA). The analytical conditions and the multiple reaction monitoring (**MRM**) transition for selected sulfur compounds were followed as described by Jo et al. (2018). Dwell times were set to ensure 3-3.1 cycles over a peak. The experiments were performed in triplicate at each time point. MassHunter Qualitative and Quantitative Analysis software (Agilent Technologies Inc., Santa Clara, CA, USA) were used for data analysis. The relative concentration of each compound was calculated based on the response ratio of the quantifier ion to that of internal standard.

#### 3.6.6. Laser diffraction particle size analysis

The particle size distributions of each RTD beverage at each time point was determined using laser diffraction (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) equipped with a Hydro EV liquid sample interface (Malvern Instruments Ltd, Worcestershire, UK). Water was used as a dispersion solvent with a refractive index of 1.333, and a refractive index of 1.570 and absorbance of 0.001 was used for the beverage particles (Griffin and Griffin, 1985). Sample solutions were added to reach an obscuration level of  $10 \pm 1\%$  and stirred at 2000 rpm for the duration of the test. The instrument measured the volume fraction in each of 100 separate size classes ranging from 0.01 to 3000  $\mu$ m. The calculation of the sizes were done using the Mie light scattering model for spherical particles.

### 3.7. Statistics

A general linear mixed model was fit using the GLIMMIX procedure in SAS (version 9.4, SAS, Cary, NC). Fixed effects included protein source, protein amount, time and all interactions. A random effect was included to account for replication effects as well as repeated measures taken across time. Least squares means were used as appropriate to investigate significant interactions and main effects. Additionally, for time 0, the general linear model (GLM) procedure of SAS was used to explore the effects of protein source and protein amount and their interaction. Least squares means were used as appropriate to investigate significant interactions and main effects

## 4. RESULTS AND DISCUSSION

The results of the proximate analysis following processing are shown in Table 2.3. In general, the protein and casein contents of the beverages were as expected. Small amounts of variability are due to differences in compositional measurement of the liquid dairy ingredients by FTIR during formulation, while the final beverage compositions were confirmed by Kjeldahl nitrogen and Mojonnier fat extraction. No coliforms were detected in beverages at any time point. The aerobic plate counts for the beverages were also < 10 CFU/mL throughout 8 weeks at 4°C storage.

### 4.1. Sensory analysis

#### 4.1.1. Overall aroma

The overall orthonasal aroma impact of the beverages was impacted by time and protein amount ( $P < 0.05$ ) (Table 2.4) (Figure 2.2). Overall aroma impact decreased from time 0 through

4 weeks at 4°C. Aroma impact was higher in 6.3% protein beverages compared to 10.5% protein beverages across 8 weeks storage at 4°C (Figure 2.3).

#### 4.1.2. Sweet aromatic/vanillin flavor

The main effects of protein content (prot) and protein blend (trt) accounted for the majority of the variance in the beverages (Table 2.4). At time 0, the lower protein beverages and beverages with lower amounts of serum protein as the percentage of true protein had higher sweet aromatic/vanillin scores compared with those with higher amounts of protein or higher amounts of serum protein as a percentage of true protein ( $P<0.05$ ) (Figure 2.4). The interaction of protein amount and time was significant ( $P<0.05$ ). Sweet aromatic/vanillin flavor decreased in all beverages with storage time at 4°C (Figure 2.5) with larger decreases in the lower protein beverages compared to the higher protein beverages.

#### 4.1.3. Cooked/milky and sulfur/eggy flavors

The main effects of protein blend (trt) followed by protein amount (prot), and time explained the majority of the variance in cooked/milky intensity in the beverages ( $P<0.05$ ) (Table 2.4). Beverages with a higher amount of serum protein as a percentage of true protein had higher intensities of cooked/milky flavor (Figure 6). The interaction of time and protein amount was also significant for cooked/milky flavor ( $P<0.05$ ). In contrast, sulfur/eggy flavor was not impacted by protein amount ( $P>0.05$ ) (Table 2.4). Protein blend (trt) and the storage time were the two main factors which affected sulfur/eggy flavor intensity ( $P<0.05$ ) (Table 2.4). Beverages with higher amounts of serum protein as a percentage of true protein had higher sulfur/eggy flavor intensities at time 0 (Figure 2.8). Sulfur/eggy flavor intensities decreased with storage time (Figure 2.9) and were not impacted by protein concentration ( $P>0.05$ ) (Table 2.4). It has been well documented that sulfur/eggy flavors in UHT and ultrapasteurized milk decrease with

storage time (Jo et al., 2018; Lee et al., 2017; Mehta, 1980; Rerkrai et al., 1987; Steely, 1994). Dunkley and Stevenson (1987) and Hansen et al. (1974) also reported that cooked flavor intensity decreased in milk and dairy beverages with storage time which is consistent with our results (Figure 2.7).

#### 4.1.4. Astringency

Beverage astringency was primarily impacted by protein amount and protein blend (Table 2.4). Beverages with higher protein and higher amounts of serum protein as a percentage of true protein had higher astringency (Figure 2.10). There were no significant interactions between any main effects for astringency ( $P>0.05$ ).

There was a statistically significant effect of time on the astringency of the beverages ( $P<0.05$ ) (Table 2.4), however the increase observed was from a mean astringency intensity of 2.7 at week 0 to a mean astringency intensity of 2.9 at week 8. While statistically significant, this increase would not be practically significant. However increases over time could be attributed to further protein network formation and aggregation during storage as previously seen by Li et al. (2018). Higher heat treatments such as UHT have been shown to increase the astringency of beverages (Lee et al., 2017; Li et al., 2018). This is likely due to the increased denaturation of the whey proteins under more intense heat treatments leading to attachment to various parts of the oral mucosa and increasing the friction within the mouth.

Much of the previous research on the astringency of protein beverages has focused on low pH whey protein beverages due to their excessive astringency (Andrewes et al., 2011; Beecher et al., 2008; Sano et al., 2005; Vardhanabhuti et al., 2010). It has been purported that in an acidic environment, the positive charge on whey proteins may interact with negatively charged salivary proteins causing drying and astringency (Beecher et al., 2008). Beecher et al.

(2008) showed that beverages at low pH (3.4) had a significantly higher astringency than the same beverages at pH 6.8. Another theory which could also explain the astringency of whey protein beverages which are not acidic may be that denatured  $\beta$ -lactoglobulin binds to oral epithelial cells increasing the drying sensation of whey proteins (Bull et al., 2017; Ye et al., 2012). Our results support the protein-mucoadhesion theory given that higher levels of serum protein led to higher astringency in a neutral pH beverage. However, due to the complexity of astringency it is possible that multiple mechanisms of action could be responsible depending on the pH, and the specific protein causing astringency.

#### 4.1.5. Viscosity

Protein amount had the largest impact on sensory perception of beverage viscosity followed by protein blend and time (Table 2.4). Higher protein beverages and beverages with higher amounts of serum protein had greater viscosity than lower protein beverages or beverages with lower amounts of serum protein as a percentage of true protein (Figure 2.11). High protein beverages made from 100% MCC did show thermoreversible gelling which has also been previously documented (results not shown) (Amelia and Barbano, 2013). The amount of protein increased the viscosity of the beverages as expected as higher protein content will result in a higher dispersed phase volume. This increase in viscosity with higher protein content is in agreement with the results of several studies (Cheng et al., 2019; Lutz et al., 2009; Wagoner and Foegeding, 2017).

Viscosity increased over time ( $P < 0.05$ ) (Table 2.4) which was expected as secondary aggregation and protein networks formed, but increases were small, similar to those observed with astringency. For low protein beverages, viscosity averaged across treatments increased only slightly, from 2.3 at time 0 to 2.4 following 8 weeks storage at 4°C while high protein beverages

increased from 2.7 to 3.0, respectively ( $P < 0.05$ ) (results not shown). The increase in viscosity during storage has been observed in previous studies involving UHT milk products (Anema, 2017; Datta and Deeth, 2001, 2003; Deeth and Lewis, 2016). Most studies focus on the changes in viscosity as an indicator of age gelation in UHT beverages where there tends to be a sharp increase in viscosity just prior to gelation.

#### 4.2. Headspace vanillin

Storage time and protein blend followed by protein amount were responsible for the majority of the variation in the beverages (Table 2.5). These results were consistent with the sensory perception of sweet aromatic/vanillin flavor presented in the previous section. Figure 2.12 shows the headspace vanillin content of the beverages at week 0. The low (6.3% w/w) protein beverages had higher amounts of vanillin than their high protein (10.5% w/w) equivalent treatments. Beverages with lower amounts of serum protein as a percentage of true protein had higher amounts of vanillin than beverages made with higher amounts of serum protein as a percentage of true protein. With increased true protein or increased serum protein as a percentage of true protein, the decrease in headspace vanillin and sensory perception of sweet aromatic/vanillin flavor suggests that higher protein decreases instrumental analysis and sensory perception of vanillin, and that these effects are increased by serum protein more than casein. Storage time also decreased vanillin content of beverages by instrumental and sensory perception (Figures 2.4, 2.13). Decreases of vanillin with storage may be due to absorption or transfer of vanillin through the packaging as well as by additional binding of vanillin by proteins. These results are consistent with much of the previous work which demonstrated that trained panel sensory perception of vanillin flavor intensity was lower in whey protein solutions than in casein solutions (Hansen and Booker, 1996; McNeill and Schmidt, 1993). Our results also support the

conclusions of Hansen and Heinis (Hansen and Heinis, 1991) who showed that increasing the concentration of whey protein concentration in solution (0.125 to 0.5%) caused a reduction in perceived sensory vanillin flavor intensity. Li et al. (2000) reported that caseinate in an aqueous solution (2% w/v) had a higher amount of free vanillin, determined by HPLC, than whey protein isolate, showing instrumentally that whey proteins have a higher affinity for binding vanillin than casein. Another study exploring interactions of vanillin in model systems found that more free vanillin, determined by HPLC, was present in 3% solutions of sodium caseinate than in a 3% solution of bovine serum albumin, and also that 6% concentrations of the proteins decreased the levels of free vanillin more than 3% solutions (Chobpattana et al., 2002).

#### 4.3. Headspace sulfur compounds

The main effects of protein blend and time followed by protein amount accounted for the majority of the variation in hydrogen sulfide and carbon disulfide in the beverages (Table 2.6). These results were consistent with the sensory perception of sulfur/eggy flavors presented in the previous section except that protein amount did not have an effect ( $P>0.05$ ) on the sensory perception of sulfur/eggy flavors. Jo et al. (2019) recently demonstrated hydrogen sulfide and carbon disulfide were responsible for sulfur/eggy and cooked/burnt flavors in UP milk and that the serum protein fraction of milk protein was the source of these volatile sulfur compounds. Figures 14 and 15 show the hydrogen sulfide and carbon disulfide content of the beverages at week 0. Low protein (6.3%) beverages had lower amounts of volatile sulfur compounds than their high protein equivalent beverages. The biggest influence in initial headspace hydrogen sulfide and carbon disulfide was the protein blend (Table 2.6), consistent with the results of Jo et al. (2019). Beverages with higher amounts of serum protein as a percentage of true protein had higher amounts of hydrogen sulfide and carbon disulfide. Volatile sulfur compounds decreased

in beverages with storage time (Figures 2.16, 2.17). Previous work has also demonstrated that these compounds decreased in UP milk across 14 days of storage at 4°C (Jo et al., 2018; Steely, 1994). The specific type of packaging will also affect the rate of dissipation of cooked and sulfur flavors depending on the permeability of gases through the package (Dunkley and Stevenson, 1987; Rysstad et al., 1998). Jo et al. (2019) further demonstrated that serum proteins were the primary source of these volatile compounds in fluid milk and milk beverages (3.3% protein). Many of these sulfur compounds are formed during maillard reactions with sulfur containing amino acids during the UHT heating, so proteins with higher amounts of sulfur-containing amino acid residues, such as serum proteins, will produce more volatile sulfur compounds during heating (Al-Attabi et al., 2008; Mehta, 1980).

#### 4.4. Particle size

The main effect, protein blend (trt), was the major factor in the differences in particle size observed among the beverages (Table 7). At time 0, beverages with higher amounts of serum protein as a percentage of true protein showed larger particle size than beverages with a lower ratio of serum protein as a percentage of true protein (Figures 2.18, 2.20, and 2.22). The beverages with 50% of true protein from SPI were the only beverages where there was a significant increase in particle size associated with higher total protein content. Because many of the high protein beverages gelled at later timepoints, measurements on these beverages were not valid. To allow for statistical analysis, we selected only the low protein beverages to evaluate for particle size over the entire shelf life. Sensory panelists also noted chalkiness and graininess in the high protein 50% serum protein beverages at week 6 and week 8, which is also consistent with protein aggregation. Larger particles can indicate protein aggregation following their denaturation by high heat treatment (Anema and Li, 2003; Dalgleish, 1990, 1992). The



denaturation of the proteins, especially the serum proteins, may account for both higher astringency as well as the larger particle size in the beverages with 50% of serum protein as a percentage of true protein. However, the increased particle size could also be increasing the friction coefficients of the particles and increasing the astringency of the beverage (De Wijk and Prinz, 2006). Li et al. (2018) observed that 5% fat content UHT milks had less the astringency than skim milks processed the same way, which was attributed to an increase in lubrication between the particles and the oral mucosa by the small fat droplets. The low fat content of our beverages (0.5%) was enough to reduce the astringency by increasing lubrication between the particles and the oral mucosa. More research would be needed to elucidate if it is simply the denaturation of the proteins responsible for both the increase in particle size and the increase in astringency or if particle size alone directly influenced the astringency of the beverages.

## 5. CONCLUSION

The type of protein and amount of protein used when formulating RTD beverages has multiple effects on various physical and sensory attributed. High concentrations of protein present greater challenges in the development of a RTD beverage which will not increase in viscosity and gel over the storage time or have reduced flavor impact. Serum protein ratios of 50% at 10.5% protein produced a thicker, less stable beverage that gelled prior to 8 weeks of storage. These same beverages also had lower levels of vanillin and higher levels of sulfur compounds after processing. Casein based ingredients when used in neutral pH beverages were heat stable, showing less increase in viscosity over storage compared to the beverages with high amounts of serum proteins. Vanillin was more strongly bound by serum proteins than casein. The higher sulfur content of serum proteins produced more hydrogen sulfide and carbon disulfide than beverages produced with casein. From the perspective of a beverage manufacturer, using

MCC as the protein source is close to ideal given the high heat stability, fewer sulfur and cooked flavors, lower astringency and lower binding of flavorings. This study establishes some of the key factors which contribute to the final sensory and physicochemical properties of RTD beverages, more research is needed on this subject, especially exploring the type of heat treatment used, the use of powdered protein ingredients and higher protein amounts.

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Table 2.1. Target experimental treatments and protein concentrations for vanilla flavored protein beverages from five different protein blends (trt) (Table 2.2) and two protein concentrations (prot) shown as percentage of the total protein (w/w).

Protein Concentration (prot) (% w/w total weight)	Treatment (trt)	Protein Source (% w/w of total protein)		
		MCC	MPC	SPI
Low 6.3	100 MCC	100	0	0
	100 MPC	0	100	0
	18/82 SPI/MCC	82	0	18
	50/50 SPI/MCC	50	0	50
	50/50 SPI/MPC	0	50	50
High 10.5	100 MCC	100	0	0
	100 MPC	0	100	0
	18/82 SPI/MCC	82	0	18
	50/50 SPI/MCC	50	0	50
	50/50 SPI/MPC	0	50	50

Table 2.2. Formulations of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (prot) shown as percent w/w. Compositions of the ingredients used for calculations shown in footnote.

Protein Concentration	Treatment (trt)	Water	SPI <sup>1</sup>	MPC <sup>2</sup>	MCC <sup>3</sup>	Lactose <sup>5</sup>	Cream <sup>4</sup>	Carrageenan	Cellulose Gel	Dipotassium Phosphate	Sucralose	Flavor
Low	100 MCC	45.55	0.00	0.00	52.47	0.38	0.81	0.015	0.40	0.15	0.03	0.20
	100 MPC	55.96	0.00	41.89	0.00	0.39	0.97	0.015	0.40	0.15	0.03	0.20
	18/82 SPI/MCC	49.92	5.07	0.00	42.95	0.41	0.86	0.015	0.40	0.15	0.03	0.20
	50/50 SPI/MCC	57.53	14.09	0.00	26.18	0.46	0.94	0.015	0.40	0.15	0.03	0.20
	50/50 SPI/MPC	62.69	14.09	20.94	0.00	0.46	1.03	0.015	0.40	0.15	0.03	0.20
High	100 MCC	11.04	0.00	0.00	87.42	0.18	0.56	0.015	0.40	0.15	0.03	0.20
	100 MPC	28.27	0.00	69.90	0.00	0.19	0.84	0.015	0.40	0.15	0.03	0.20
	18/82 SPI/MCC	18.19	8.47	0.00	71.67	0.23	0.64	0.015	0.40	0.15	0.03	0.20
	50/50 SPI/MCC	30.90	23.52	0.00	43.69	0.31	0.79	0.015	0.40	0.15	0.03	0.20
	50/50 SPI/MPC	39.51	23.51	34.95	0.00	0.31	0.93	0.015	0.40	0.15	0.03	0.20

<sup>1</sup>SPI Composition (0.15% fat, 22.29% protein, 0.52% lactose) (% w/w).

<sup>2</sup>MPC Composition (0.20% fat, 15.00% protein, 0.68% lactose) (% w/w).

<sup>3</sup>MCC Composition (0.30% fat, 12.86% protein, 0.56% lactose) (% w/w).

<sup>4</sup>Cream Composition (42.48% fat, 1.75% protein, 2.82% lactose) (% w/w).

<sup>5</sup>Lactose (95% purity with maximum of 5% moisture) (% w/w).

Table 2.3. Proximate analysis of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (prot) (Table 2.1) shown as mean percentage w/w.

Protein	Treatment	Fat	Total Nitrogen	Non-Casein Nitrogen	Non-Protein Nitrogen	True Protein	Casein	Total Solids	Lactose	
Low	6.3%	100 MCC	0.38±0.01	7.16±0.02	0.67±0.06	0.07±0.01	7.10±0.02	6.43±0.04	9.63±0.03	0.74±0.02
		100 MPC	0.51±0.01	6.22±0.02	1.12±0.07	0.05±0.01	6.18±0.02	5.06±0.09	8.71±0.05	0.69±0.04
		18/82 SPI/MCC	0.39±0.01	7.00±0.02	1.55±0.02	0.07±0.01	6.92±0.02	5.37±0.03	9.48±0.01	0.78±0.02
		50/50 SPI/MCC	0.43±0.01	6.82±0.10	3.18±0.07	0.07±0.01	6.75±0.10	3.57±0.03	9.24±0.09	0.85±0.02
		50/50 SPI/MPC	0.49±0.01	6.39±0.11	3.44±0.14	0.06±0.01	6.33±0.11	2.89±0.02	8.84±0.15	0.83±0.03
High	10.5%	100 MCC	0.27±0.04	11.89±0.04	0.94±0.11	0.08±0.01	11.81±0.05	10.87±0.07	14.78±0.03	0.79±0.04
		100 MPC	0.52±0.01	10.44±0.07	1.77±0.20	0.06±0.01	10.37±0.08	8.60±0.13	13.18±0.12	0.70±0.07
		18/82 SPI/MCC	0.33±0.02	11.63±0.07	2.28±0.27	0.10±0.01	11.53±0.08	9.25±0.20	14.54±0.02	0.85±0.05
		50/50 SPI/MCC	0.38±0.01	11.23±0.39	4.98±0.13	0.10±0.01	11.13±0.37	6.14±0.26	13.98±0.28	0.96±0.04
		50/50 SPI/MPC	0.51±0.01	10.47±0.17	5.52±0.18	0.09±0.01	10.39±0.17	4.86±0.01	13.25±0.08	0.92±0.05

Table 2.4. Analysis of variance type III sum of squares F statistics for descriptive sensory profiles of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (prot) (Table 2.1) across 8 weeks storage at 4°C (time).

Factor	Overall	Sweet				
	aroma	aromatic/vanillin	Cooked/milky	Sulfur/eggy	Astringency	Viscosity
Trt	15.5*	145.6*	89.1*	160.1*	125.6*	51.1*
Prot	11.3*	85.3*	18.7*	1.56	201.4*	121.08*
Trt*prot	0.13	0.41	0.70	0.62	0.52	5.56*
Time	9.2*	31.3*	22.0*	151.9*	6.46*	7.81*
trt*time	0.88	0.55	1.60	11.1*	0.75	1.43
Time*prot	4.52*	3.51*	2.83*	1.71	0.40	1.21
Trt*time*prot	0.24	0.27	0.70	0.95	0.25	0.76

\*P<0.05

Metallic and sweet taste were not included as these mean attributes did not change by more than +/- 0.2 across treatment, protein amount, or storage time.

Table 2.5. Analysis of variance type III sum of squares F statistics for volatile compound analysis of vanillin by SPME GC-MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (prot) (Table 2.1) across 8 weeks storage at 4°C (time).

Factor	Vanillin ppb
Trt	969.7*
Prot	799.6*
Trt*prot	290.4*
Time	1788.1*
Trt*time	88.31*
Time*prot	223.5*
Trt*time*prot	53.33*

\*P<0.05

Table 2.6. Analysis of variance type III sum of squares F statistics for volatile compound analysis of hydrogen sulfide and carbon disulfide by SPME GC-MS/MS from five different protein blends (trt) and two protein concentrations (prot) (Table 2.1) across 8 weeks storage at 4°C (time).

Factor	Hydrogen sulfide	Carbon disulfide
Trt	4207*	2861*
Prot	185.8*	148.1*
Trt*prot	7.34*	3.62
Time	4824*	5378*
Trt*time	469.5*	517.2*
Time*prot	19.58*	22.91*
Trt*time*prot	2.00*	2.15*

\*P<0.05

Table 2.7. Analysis of variance type III sum of squares F statistics for particle size ( $\mu\text{m}$ ) and specific surface area ( $\text{m}^2/\text{kg}$ ) measures of vanilla flavored protein beverages from five different protein blends (trt) (Table 2.1) across 8 weeks storage at 4°C (time).

Factor	D <sub>10</sub>	D <sub>50</sub>	D <sub>90</sub>	D <sub>[4,3]</sub>	Specific Surface Area
Trt	30.77*	14.07*	3.68	5.65	50.30*
Time	4.35*	1.15	0.70	0.52	3.10*
Trt*time	2.40*	2.85*	0.77	1.00	0.60

\*P<0.05

Only 6.3% protein beverages analyzed due to gel formation of some 10.5% protein beverages following storage at 4°C.

## Beverage Formulation

Formulation					Desired Final Composition				Ingredient Composition					
Ingredient	Min	Max	% (wt/wt)	Amount	Unit	Parameter	Min	Max	Value		Total Solids	Total Fat	Protein	Lactose
Water	0.00%	100.00%	45.55%	15.94	kg	Total Fat	0.500%	0.500%	0.500%	Water	0.00%	0.00%	0.00%	0.00%
SPI	0.00%	100.00%	0.00%	0.00	kg	Total Lactose	0.680%	0.680%	0.680%	SPI	22.96%	0.15%	22.29%	0.52%
MPC	0.00%	100.00%	0.00%	0.00	kg	Protein from SPI	0.000%	0.000%	0.000%	MPC	15.88%	0.20%	15.00%	0.68%
MCC	0.00%	100.00%	52.47%	18.36	kg	Protein From MPC	0.000%	0.000%	0.000%	MCC	12.86%	0.30%	12.00%	0.56%
Lactose	0.00%	1.00%	0.38%	133.90	g	Protein From MCC	100.00%	100.00%	100.000%	Lactose	95.00%	0.00%	0.00%	95.00%
Cream	0.00%	5.00%	0.81%	282.46	g	Total Solids			8.105%	Cream	47.05%	42.48%	1.75%	2.82%
Carrageenan	0.015%	0.015%	0.02%	5.25	g	Total Protein	6.310%	6.310%	6.310%	Carrageenan	100.00%	0.00%	0.00%	0.00%
Cellulose Gel	0.40%	0.40%	0.40%	140.00	g					Cellulose Gel	100.00%	0.00%	0.00%	0.00%
DiKphosphate	0.15%	0.15%	0.15%	52.50	g					DiKphosphate	0.00%	0.00%	0.00%	0.00%
Sucralose	0.03%	0.03%	0.03%	10.50	g					Sucralose	0.00%	0.00%	0.00%	0.00%
Flavor	0.20%	0.20%	0.20%	70.00	g					Flavor	100.00%	0.00%	0.00%	0.00%
		<b>Total</b>	100.00%	35.000										
				<b>Input batch weight</b>										

Figure 2.1. Solver used to calculate formulation for desired beverage composition. Formula results show a 35.00 kg batch of 6.3% protein 100% MCC beverage. The final composition parameters are set in the middle panel and the composition of each ingredient used in the formulation is entered into the right panel. The GRG optimizer function of Excel calculates the formula given the restrictions of the desired composition.

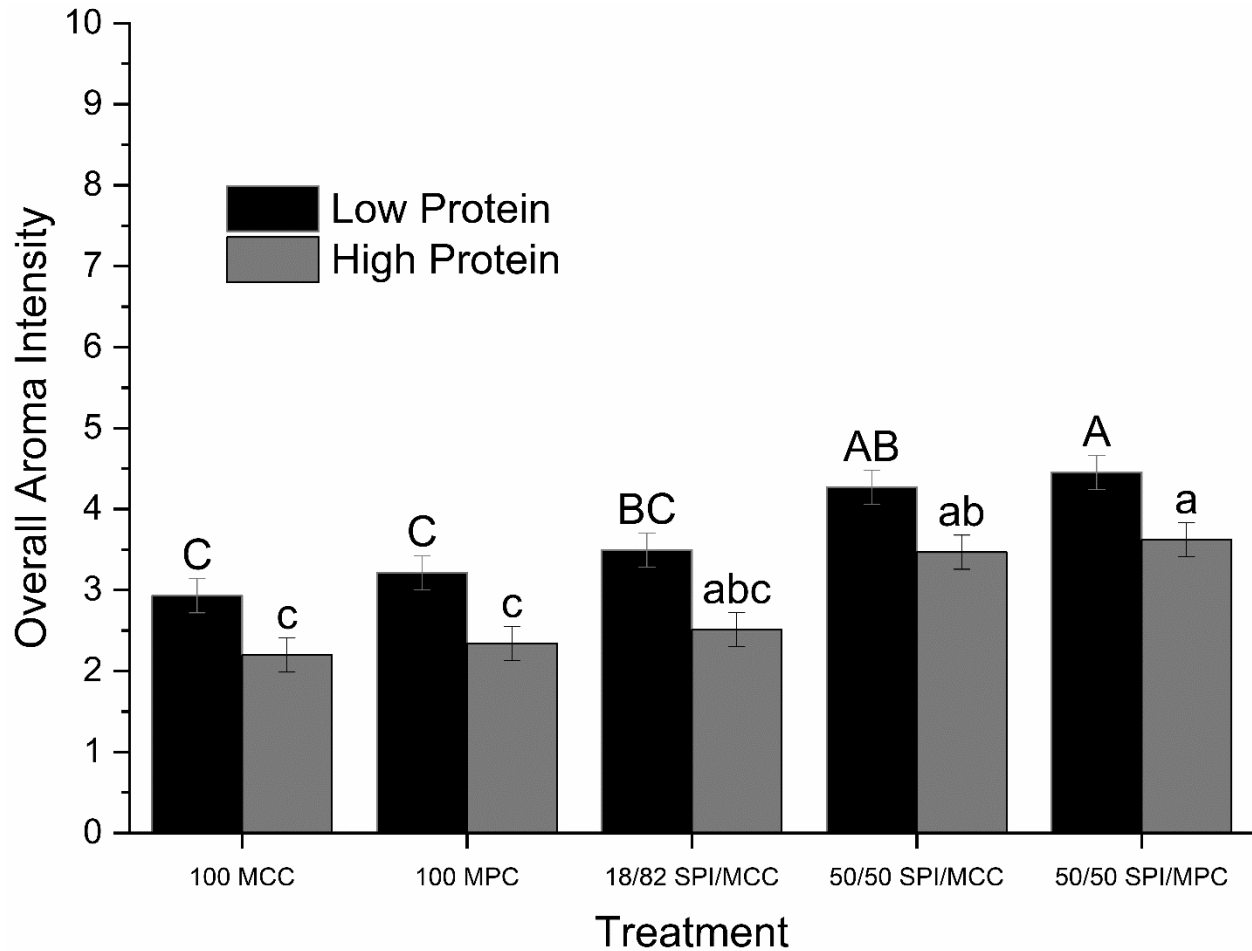


Figure 2.2. Least square means of the trained panel sensory perception of overall aroma intensity of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Lower case letters indicate differences among 6.3% protein beverages and uppercase letters indicate differences among 10.5% protein beverages ( $P < 0.05$ ). There was not a significant (trt x prot) interaction ( $P > 0.05$ ). Error bars above each bar indicate standard error of 0.220.

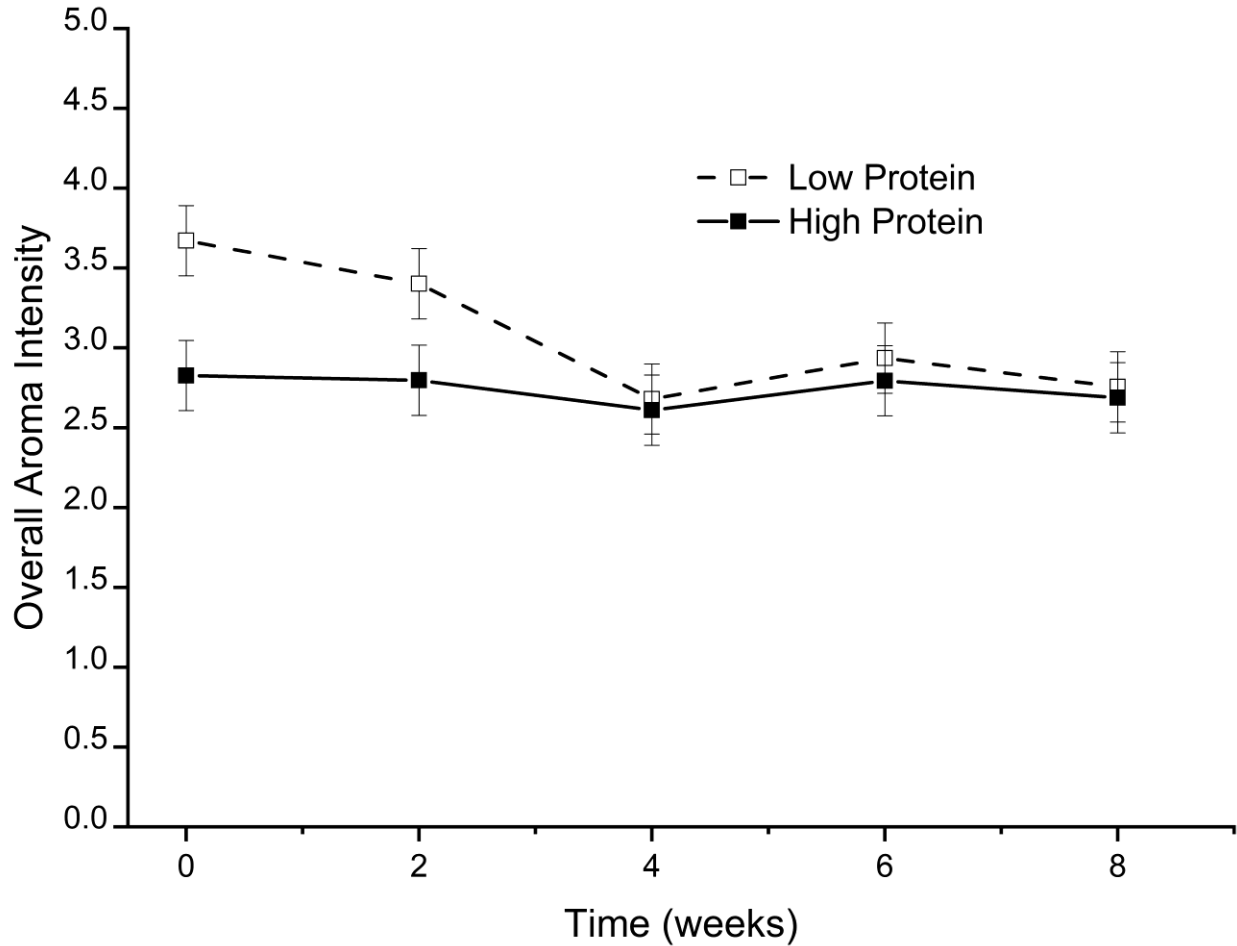


Figure 2.3. Least square means of trained panel sensory perception of overall aroma intensity of vanilla flavored protein beverages from two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. The interaction of (time x prot) was significant ( $P < 0.05$ ). Standard error 0.220.



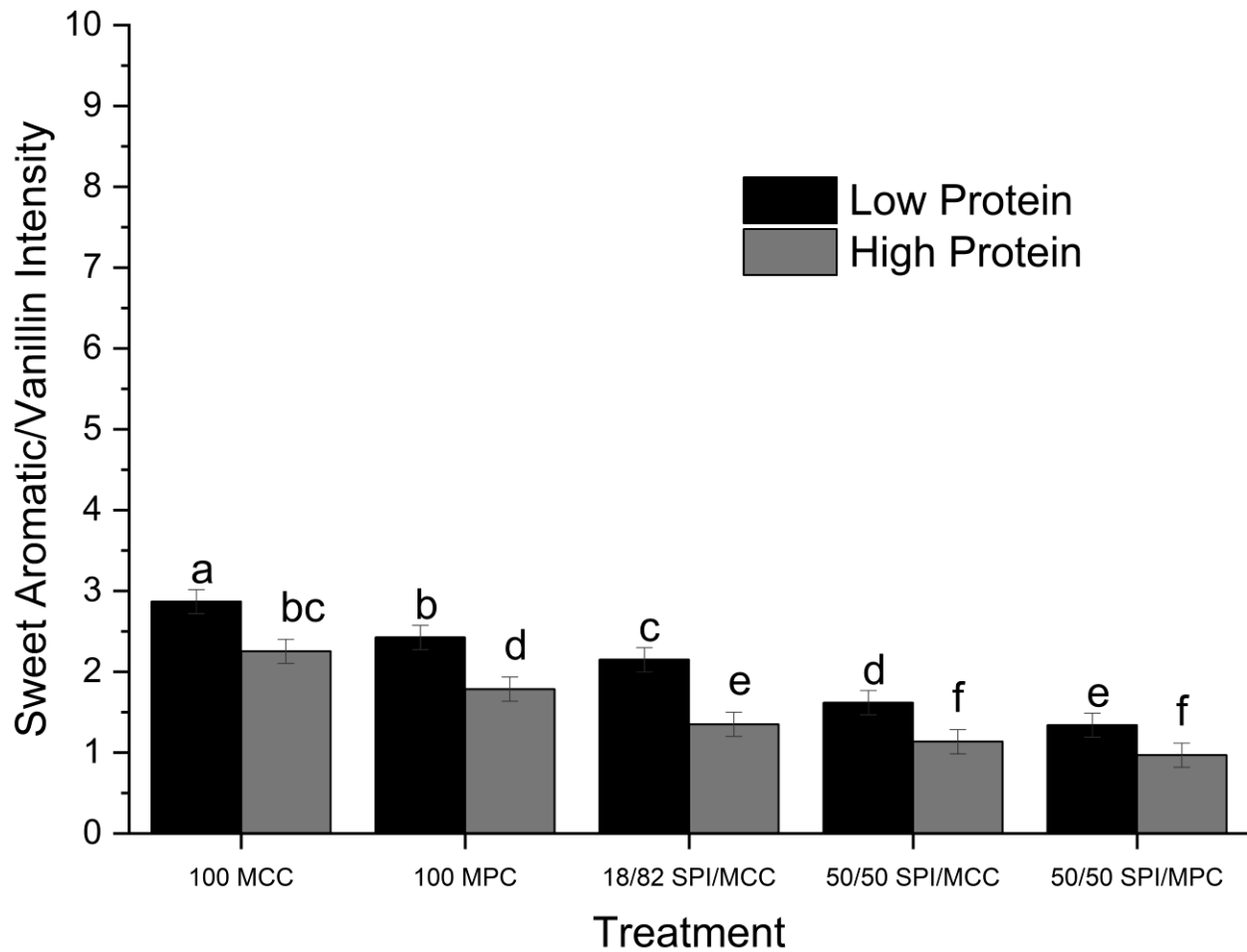


Figure 2.4. Least square means of the trained panel sensory perception sweet aromatic/vanillin flavor of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Different letters indicate differences among the beverages ( $P < 0.05$ ). Error bars above each bar indicate standard error of 0.149

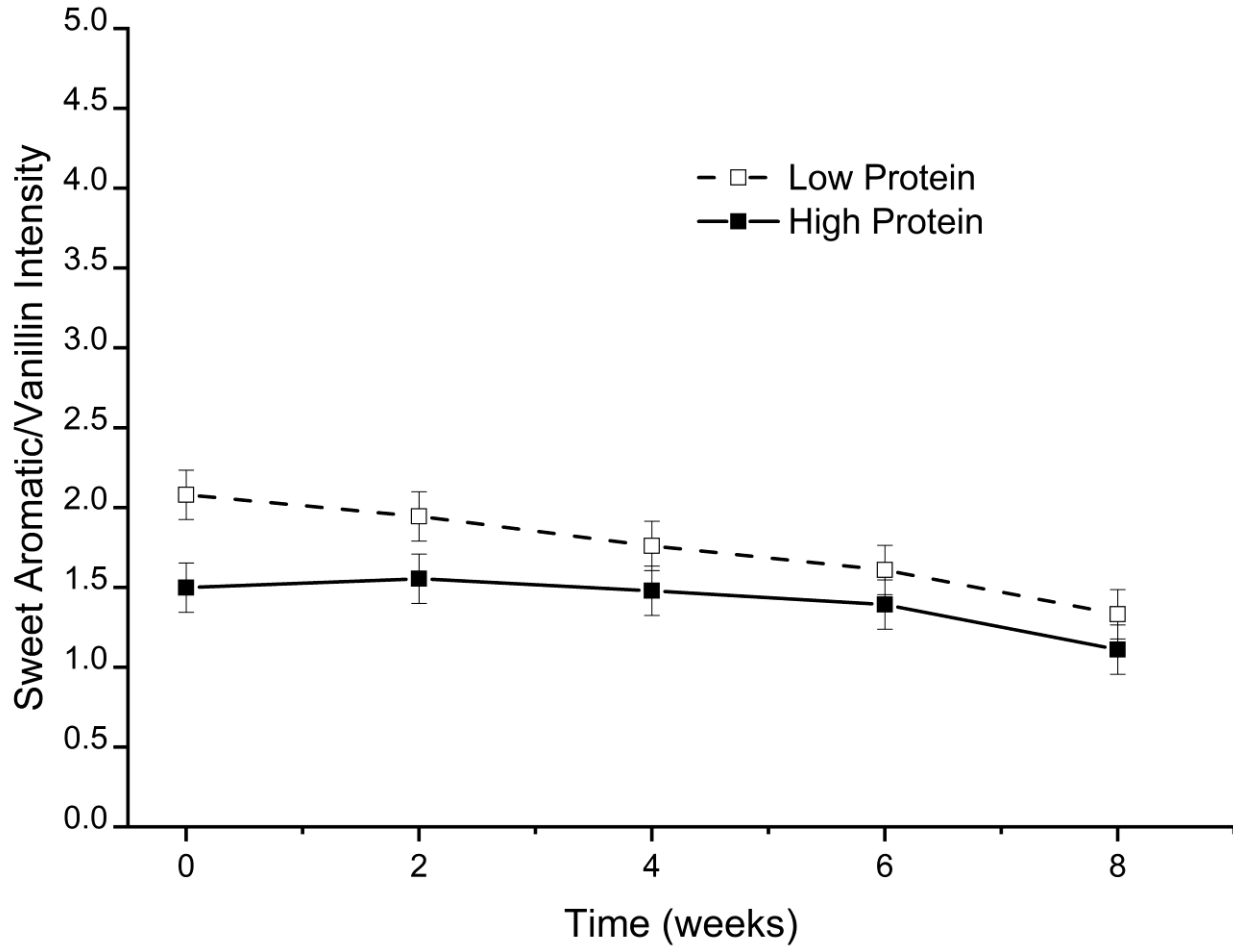


Figure 2.5. Least square means of trained panel sensory perception of sweet aromatic/vanillin flavor of vanilla flavored protein beverages from two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. The interaction of (time x prot) was significant ( $P < 0.05$ ). Standard error 0.154.

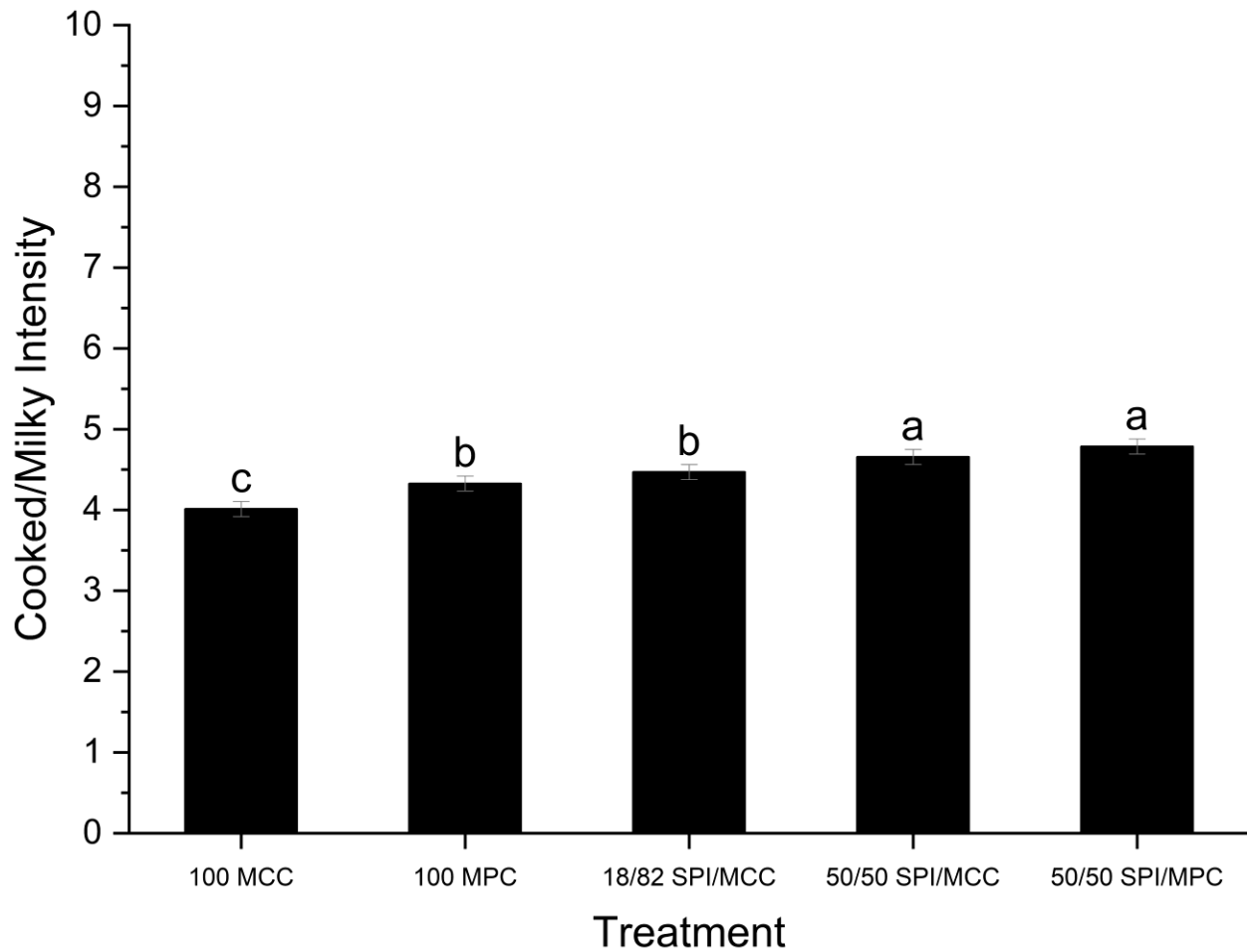


Figure 2.6. Least square means of the sensory profiles of cooked/milky intensity of vanilla flavored protein beverages from five different protein blends (trt) (Table 2.1) at time 0. The figure is the average intensity of each treatment as there was no impact of protein concentration at time 0 ( $P>0.05$ ). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Different letters indicate differences among the beverages ( $P<0.05$ ). The main effect (trt) was the only significant effect at time 0 ( $P<0.05$ ), prot and (prot x trt) were not significant ( $P>0.05$ ). Error bars above each bar indicate standard error of 0.096.

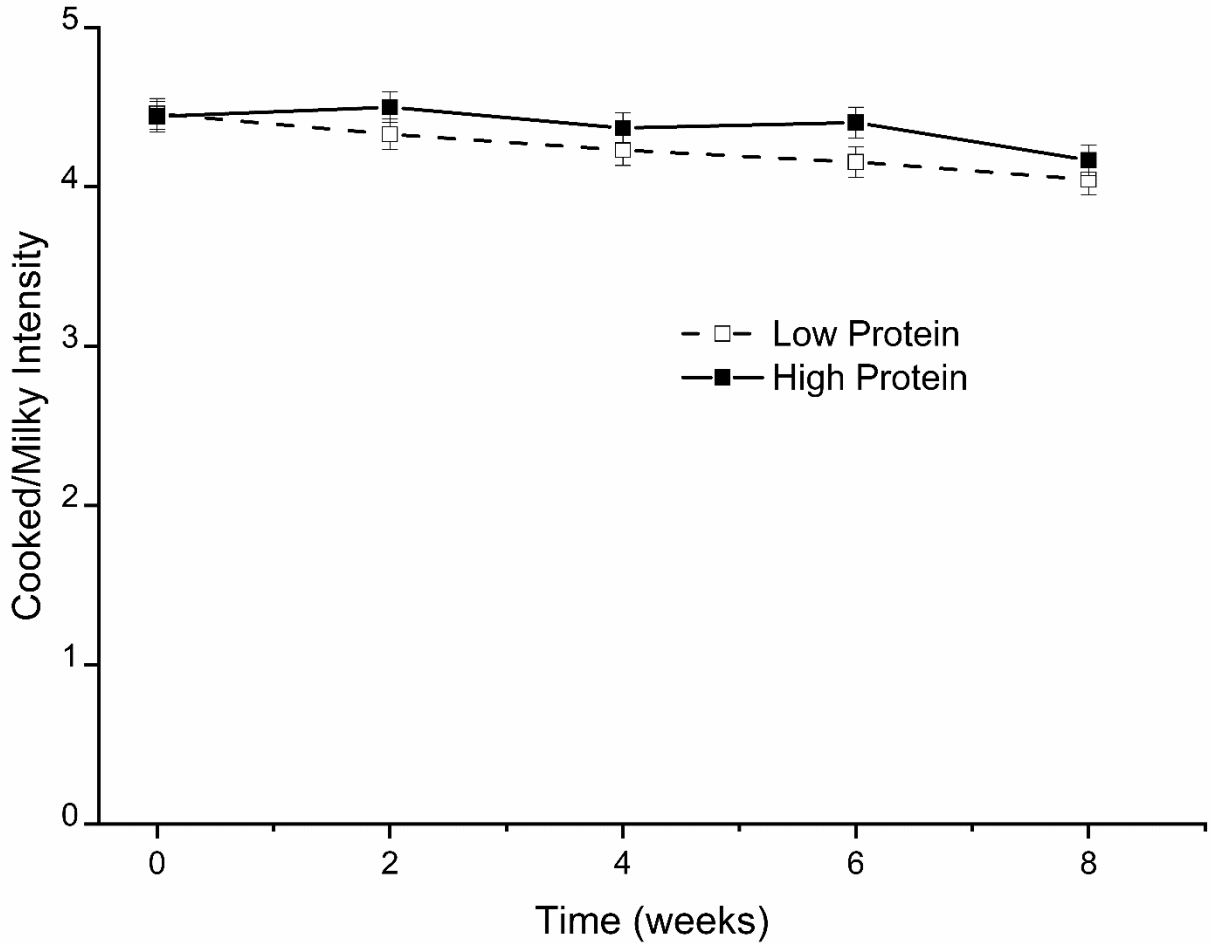


Figure 2.7. Least square means of trained panel sensory perception of cooked/milky flavor of vanilla flavored protein beverages from two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. The interaction of (time x prot) was significant ( $P < 0.05$ ). Standard error 0.096.

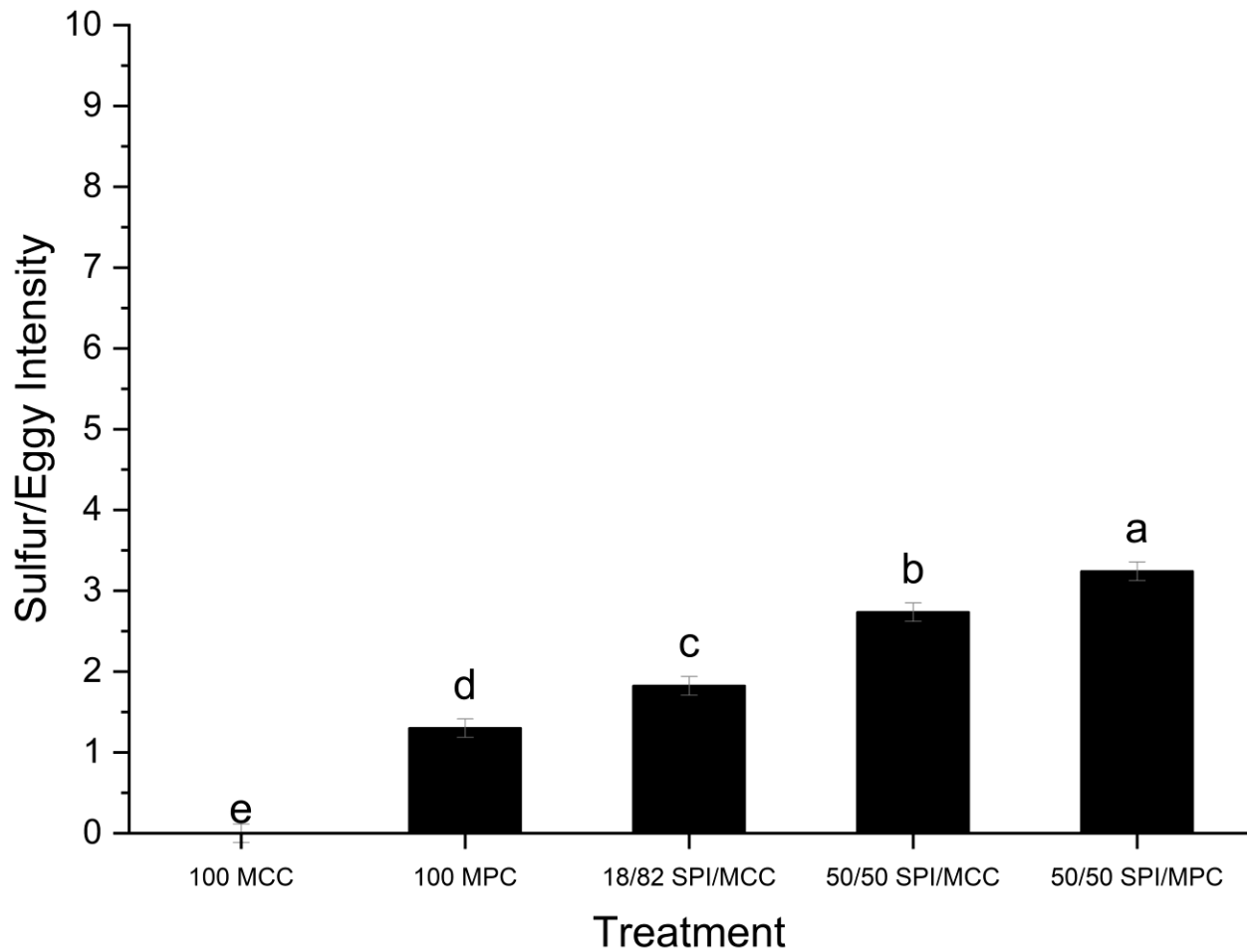


Figure 2.8. Least square means of the sensory profiles of sulfur/eggy intensity of vanilla flavored protein beverages from five different protein blends (trt) (Table 2.1) at time 0. The figure is the average intensity of each treatment as there was no effect of protein amount at time 0 ( $P>0.05$ ). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Different letters indicate differences among the beverages ( $P<0.05$ ). The main effect (trt) was the only significant effect ( $P<0.05$ ) at time 0, prot and (prot x trt) were not significant ( $P>0.05$ ). Error bars above each bar indicate standard error of 0.116.

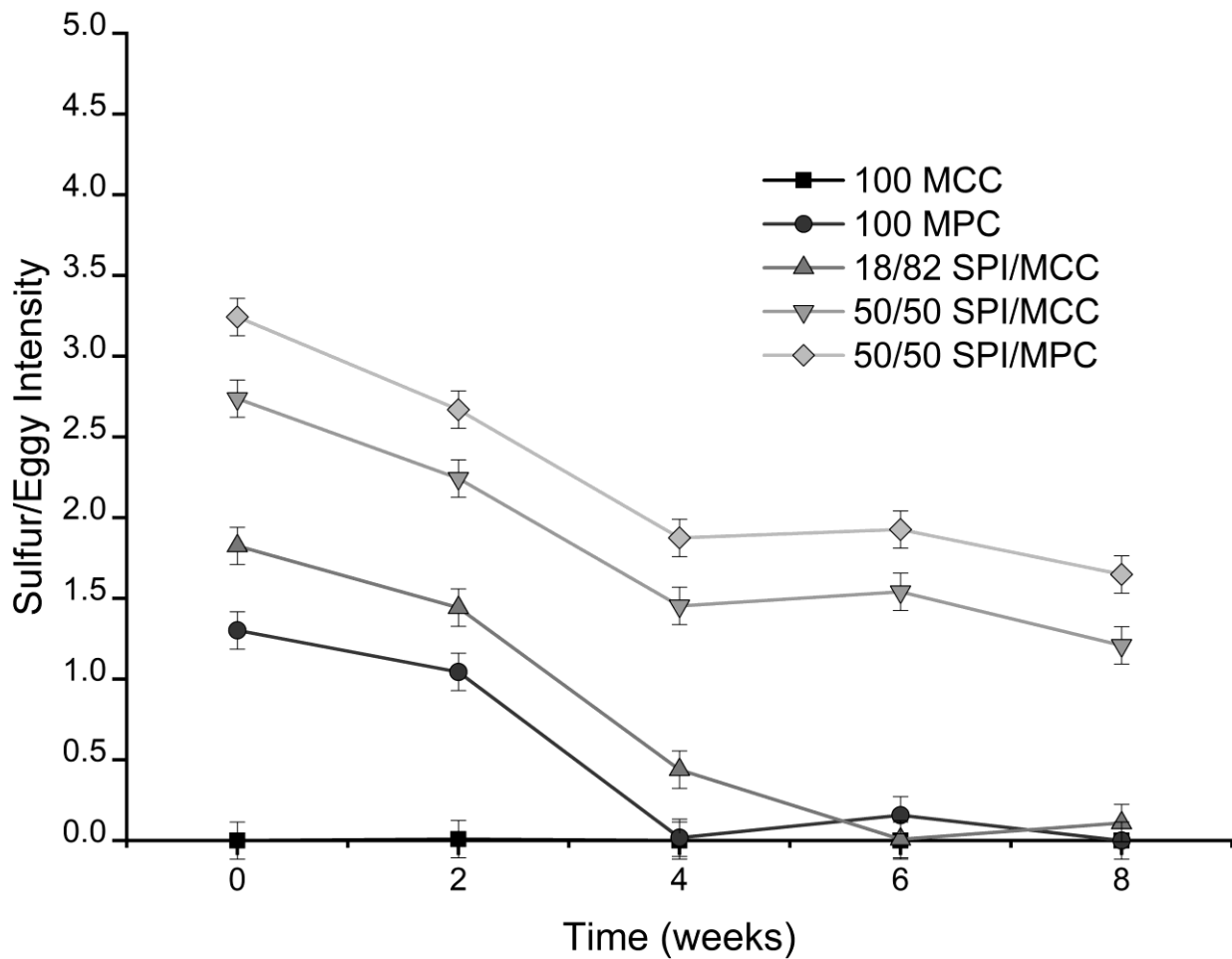


Figure 2.9. Least square means of trained panel sensory perception of sulfur/eggy flavor of vanilla flavored protein beverages from five different protein blends (trt) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. The interaction of (time x trt) was significant ( $P < 0.05$ ). Standard error 0.116.

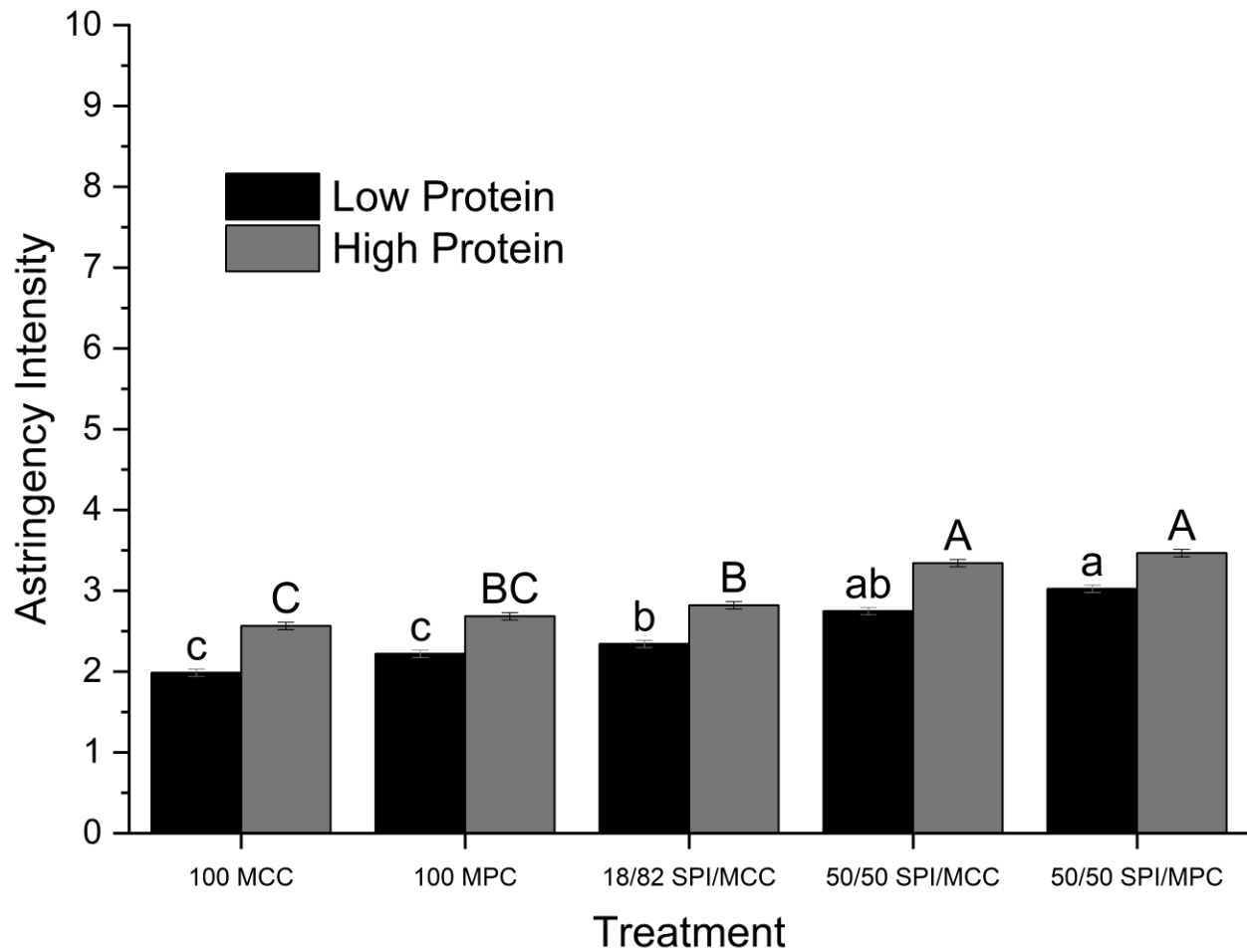


Figure 2.10. Least square means of the sensory perception of astringency of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Lower case letters indicate differences among 6.3% protein beverages and uppercase letters indicate differences among 10.5% protein beverages ( $P < 0.05$ ). There was not a significant (trt x prot) interaction ( $P > 0.05$ ). Error bars above each bar indicate standard error of 0.046.

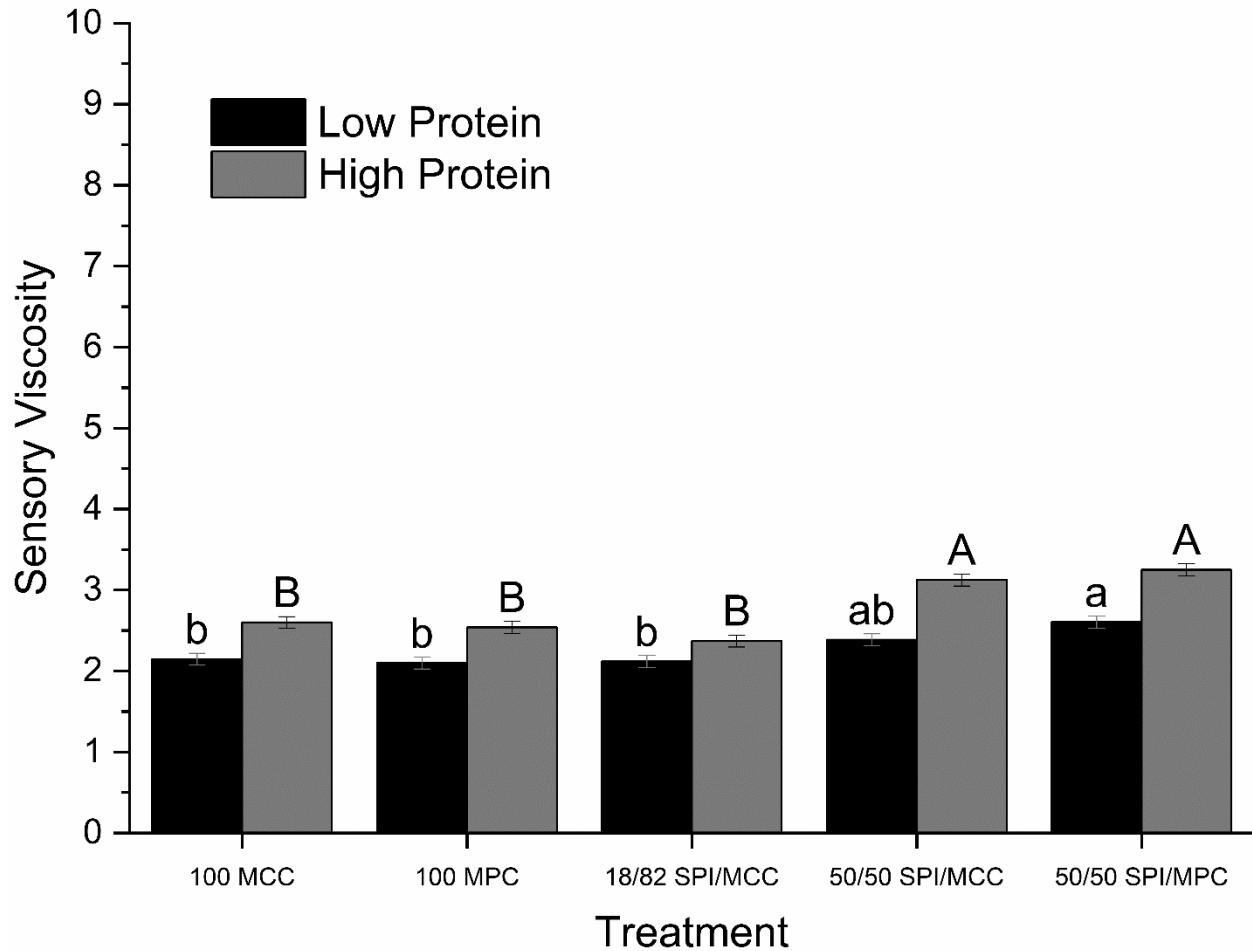


Figure 2.11. Least square means of the sensory perception of viscosity of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Lower case letters indicate differences among 6.3% protein beverages and uppercase letters indicate differences among 10.5% protein beverages ( $P < 0.05$ ). There was not a significant (trt x prot) interaction ( $P > 0.05$ ). Error bars above each bar indicate standard error of 0.052.



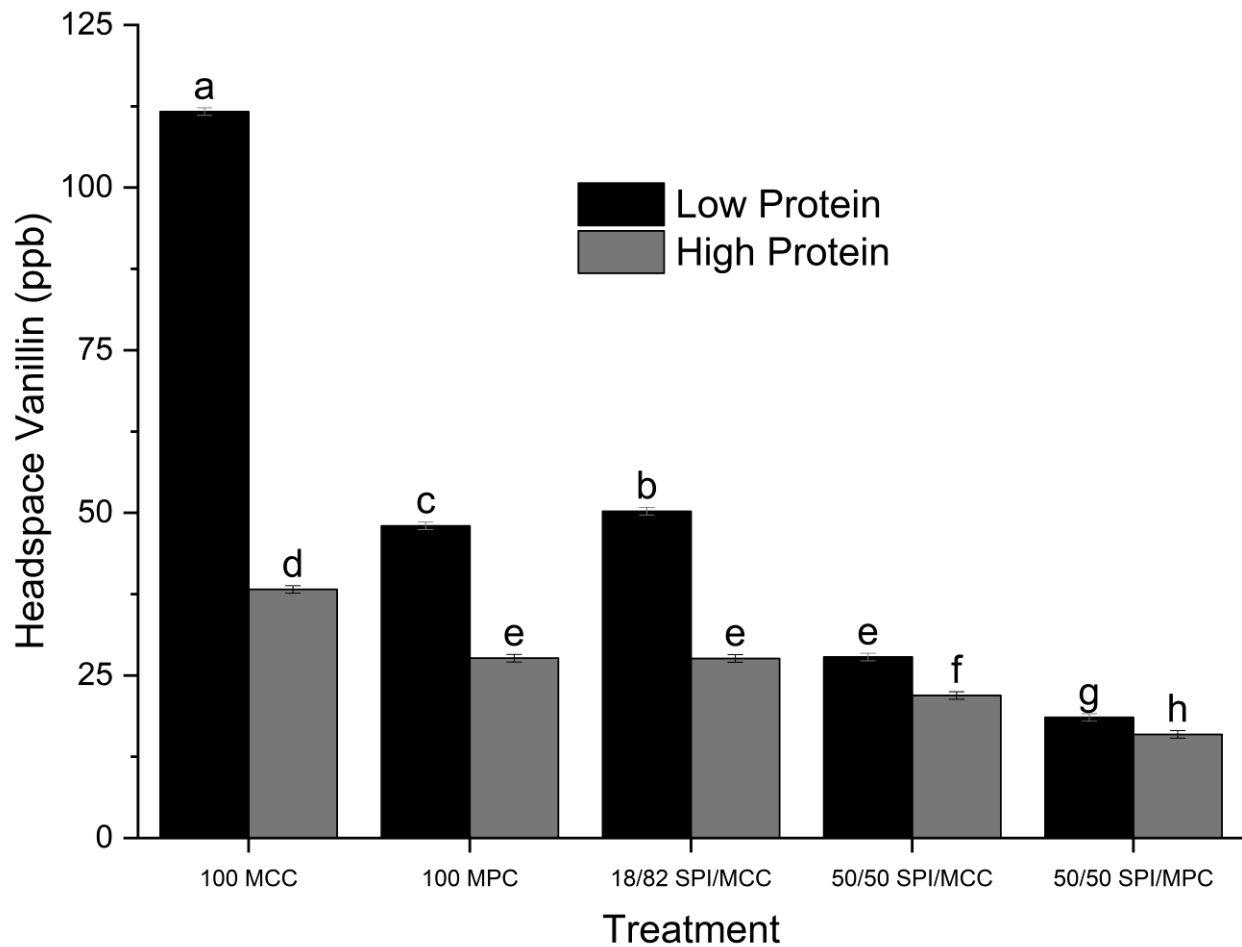


Figure 2.12. Least square means of volatile compound analysis of vanillin by SPME GC-MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Standard error 0.534. Different letters indicate differences among the beverages ( $P < 0.05$ ). Error bars above each bar indicate standard error of 0.534.

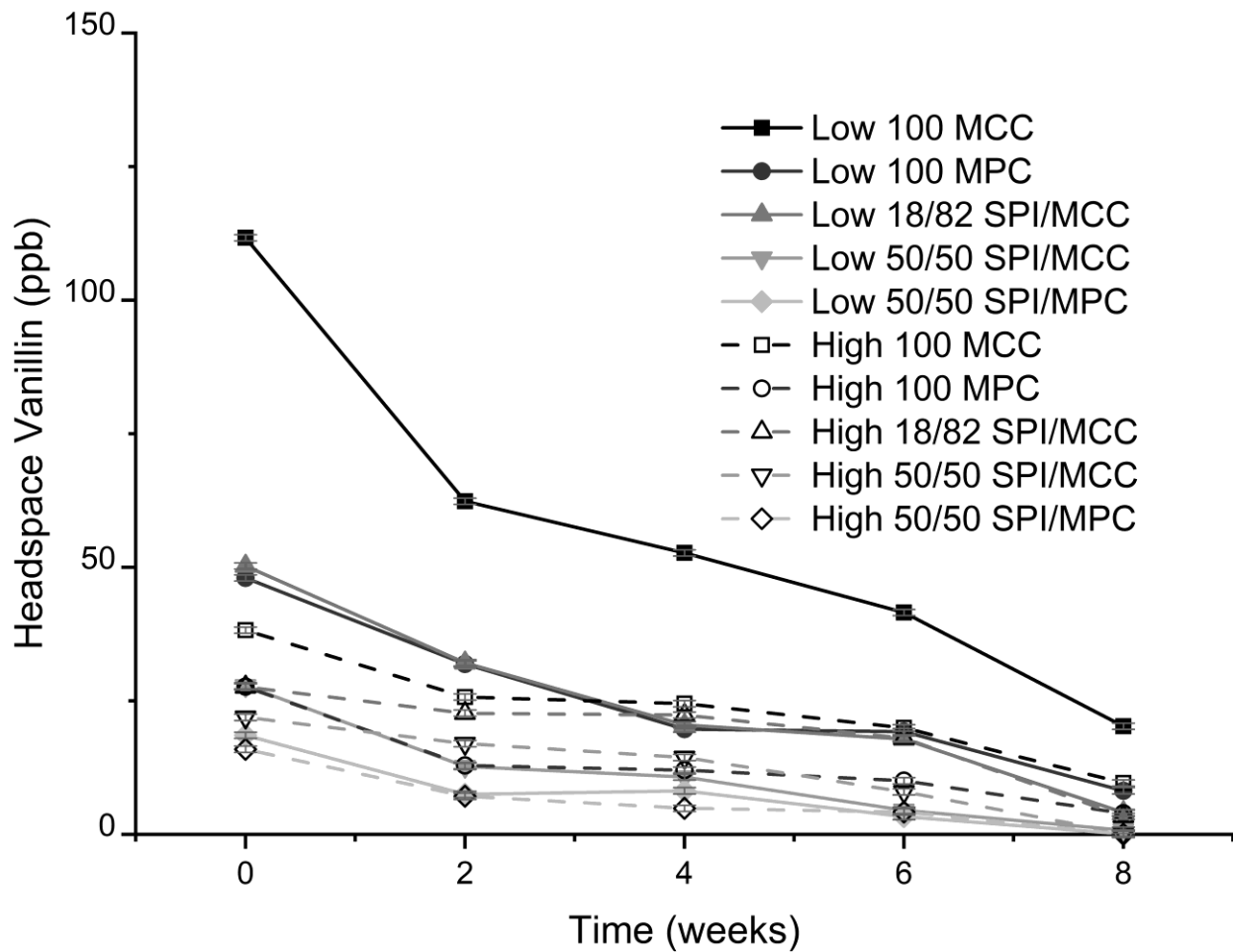


Figure 2.13. Least square means of volatile compound analysis of vanillin by SPME GC-MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks storage at 4°C (time). Standard Error 0.586.

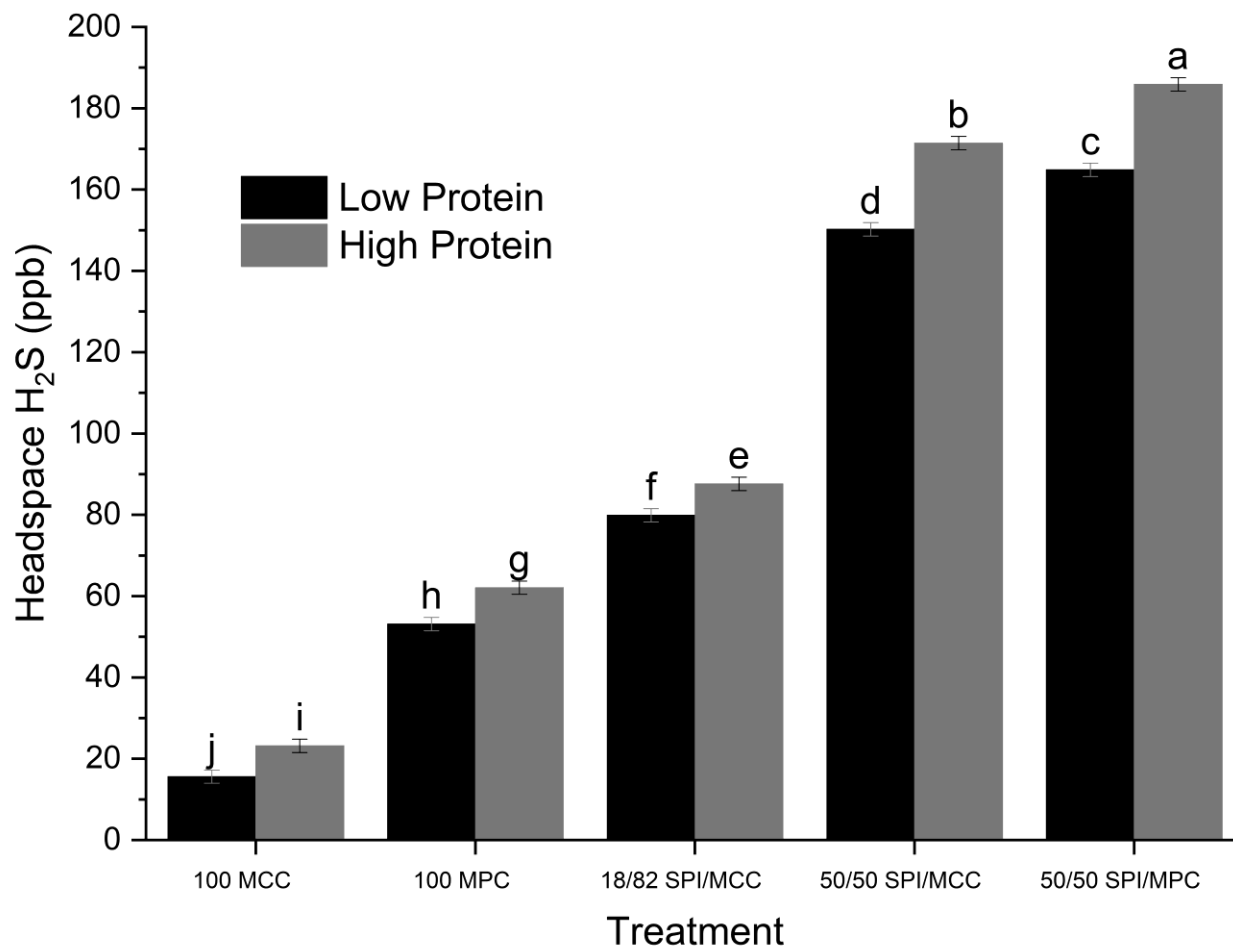


Figure 2.14. Least square means of volatile compound analysis of hydrogen sulfide by SPME GC-MS/MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Different letters indicate differences among the beverages ( $P < 0.05$ ). Error bars above each bar indicate standard error of 3.311.

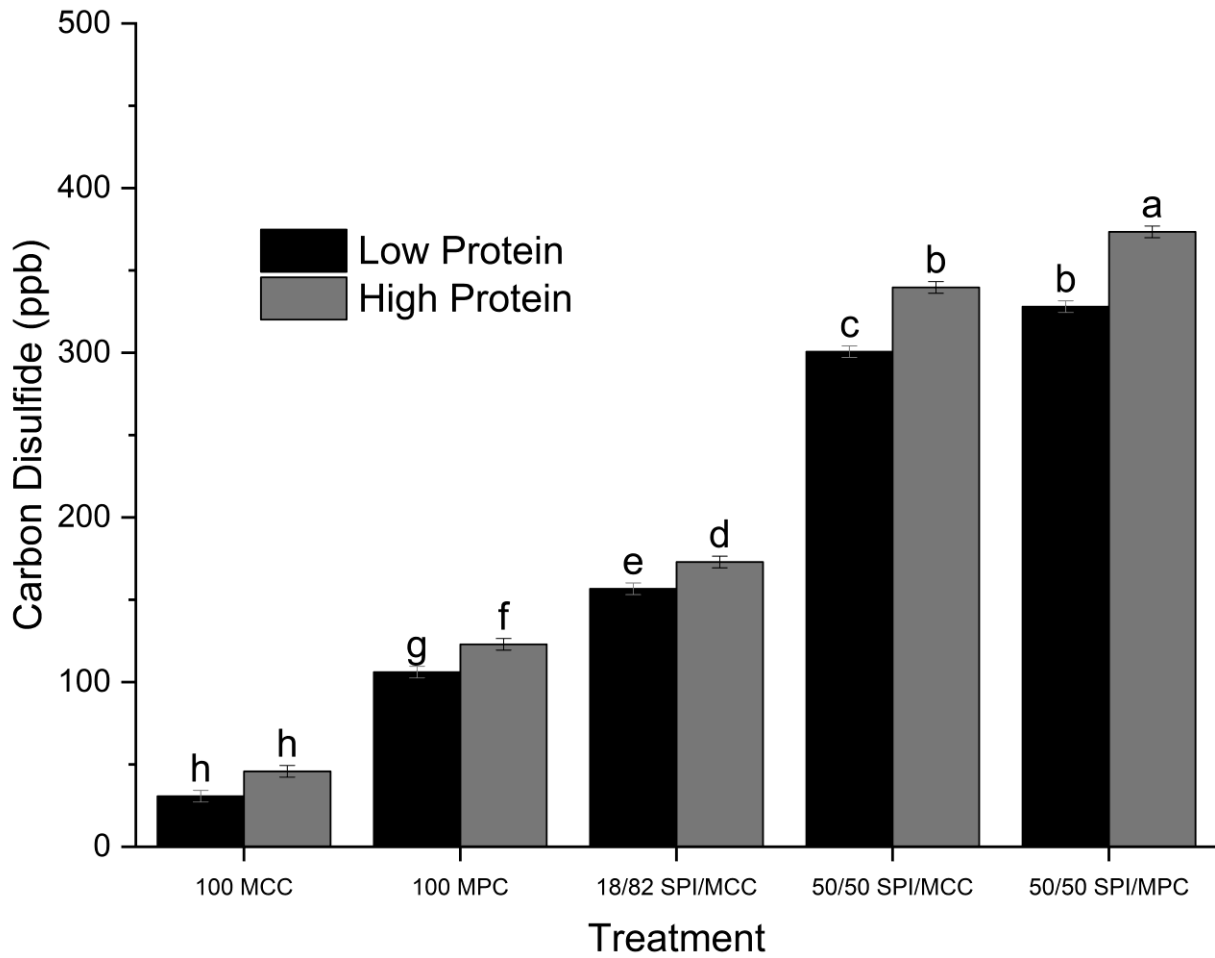


Figure 2.15. Least square means of volatile compound analysis of carbon disulfide by SPME GC-MS/MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Different letters indicate differences among the beverages ( $P < 0.05$ ). Error bars above each bar indicate standard error of 3.532.

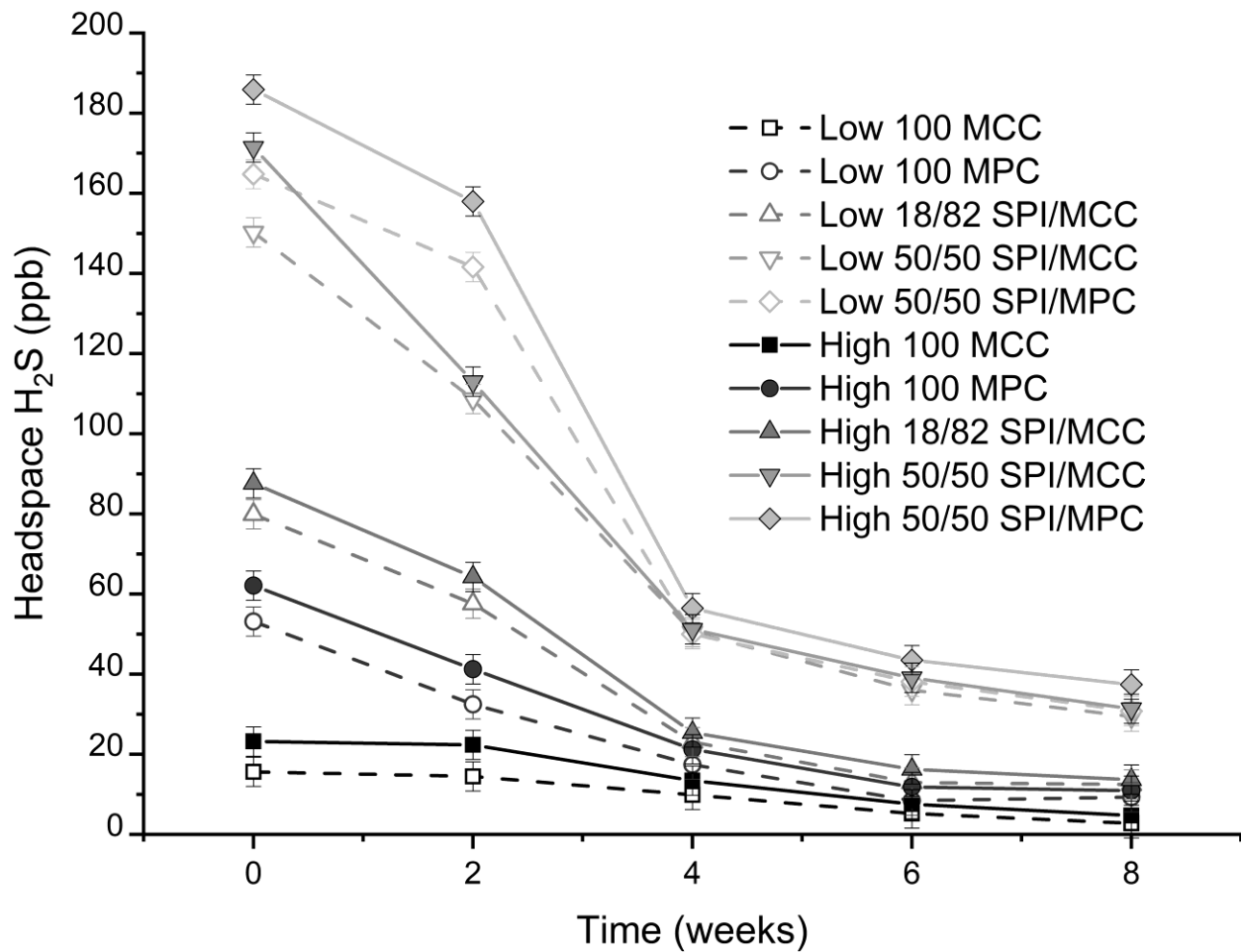


Figure 2.16. Least square means of volatile compound analysis of hydrogen sulfide by SPME GC-MS/MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks storage at 4°C (time). Standard Error 3.649.

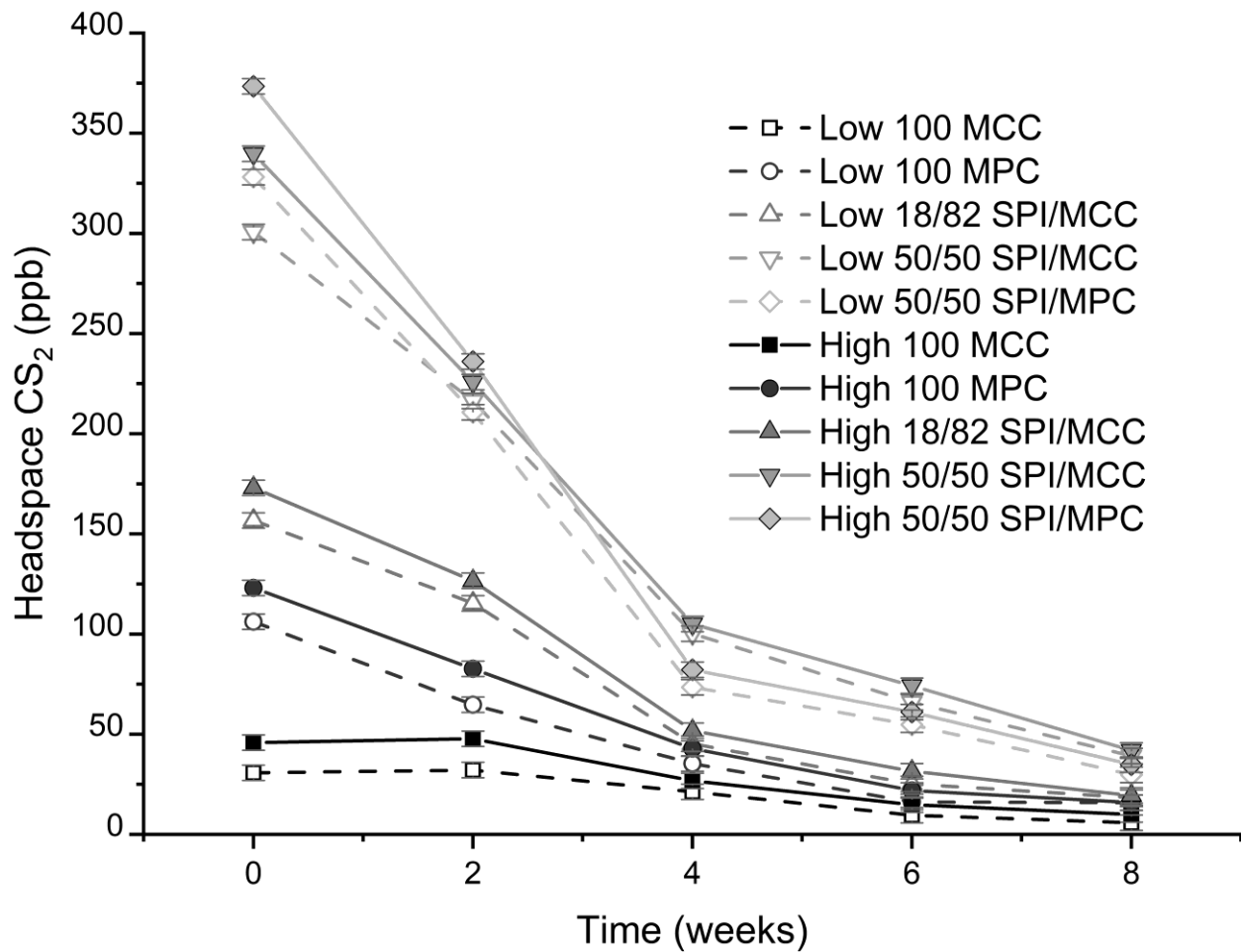


Figure 2.17. Least square means of volatile compound analysis of carbon disulfide by SPME GC-MS/MS of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks storage at 4°C (time). Standard error 3.845

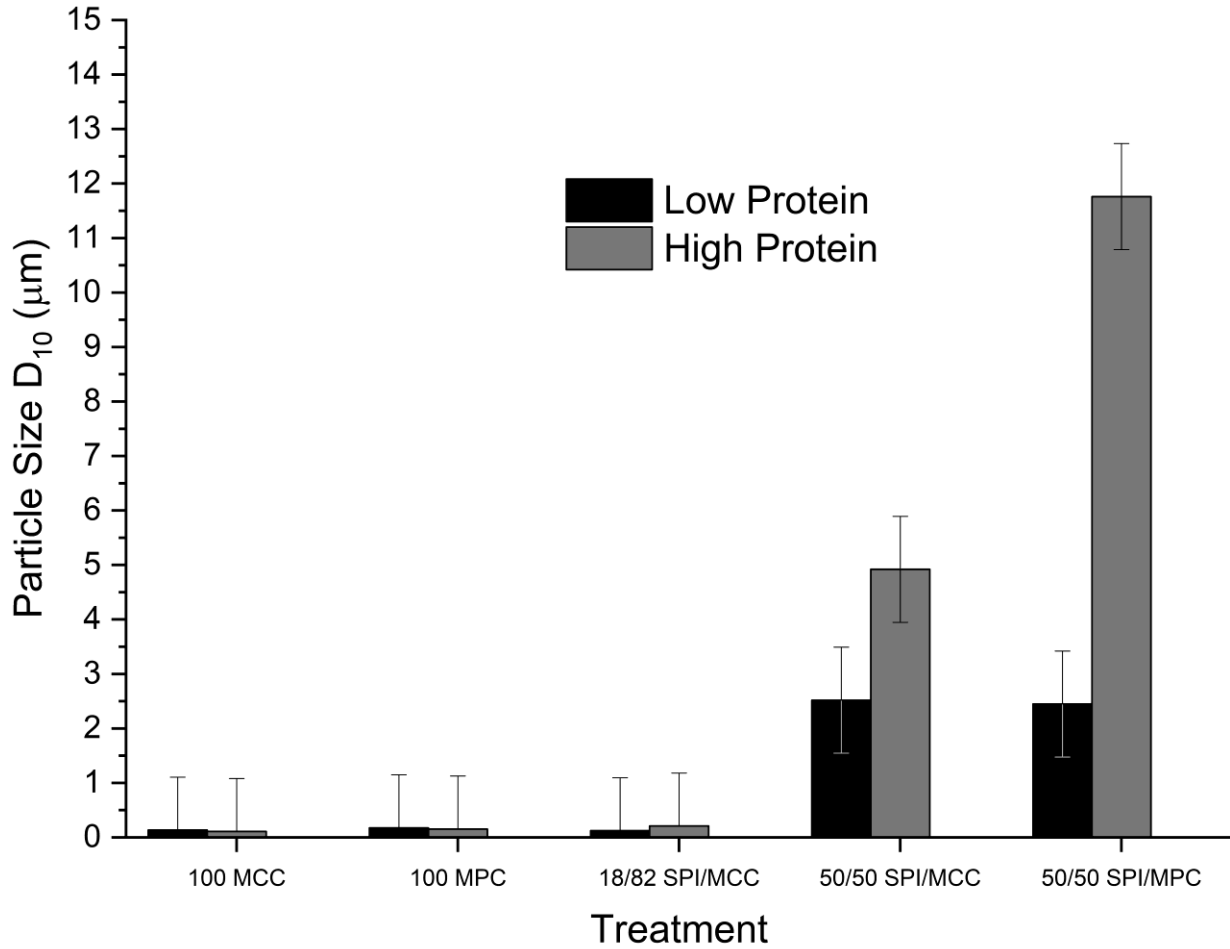


Figure 2.18. Least square means of the particle size D<sub>10</sub> (µm) of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. There was an interaction (trt x prot) ( $P < 0.05$ ). Error bars above each bar indicate standard error of 0.973.

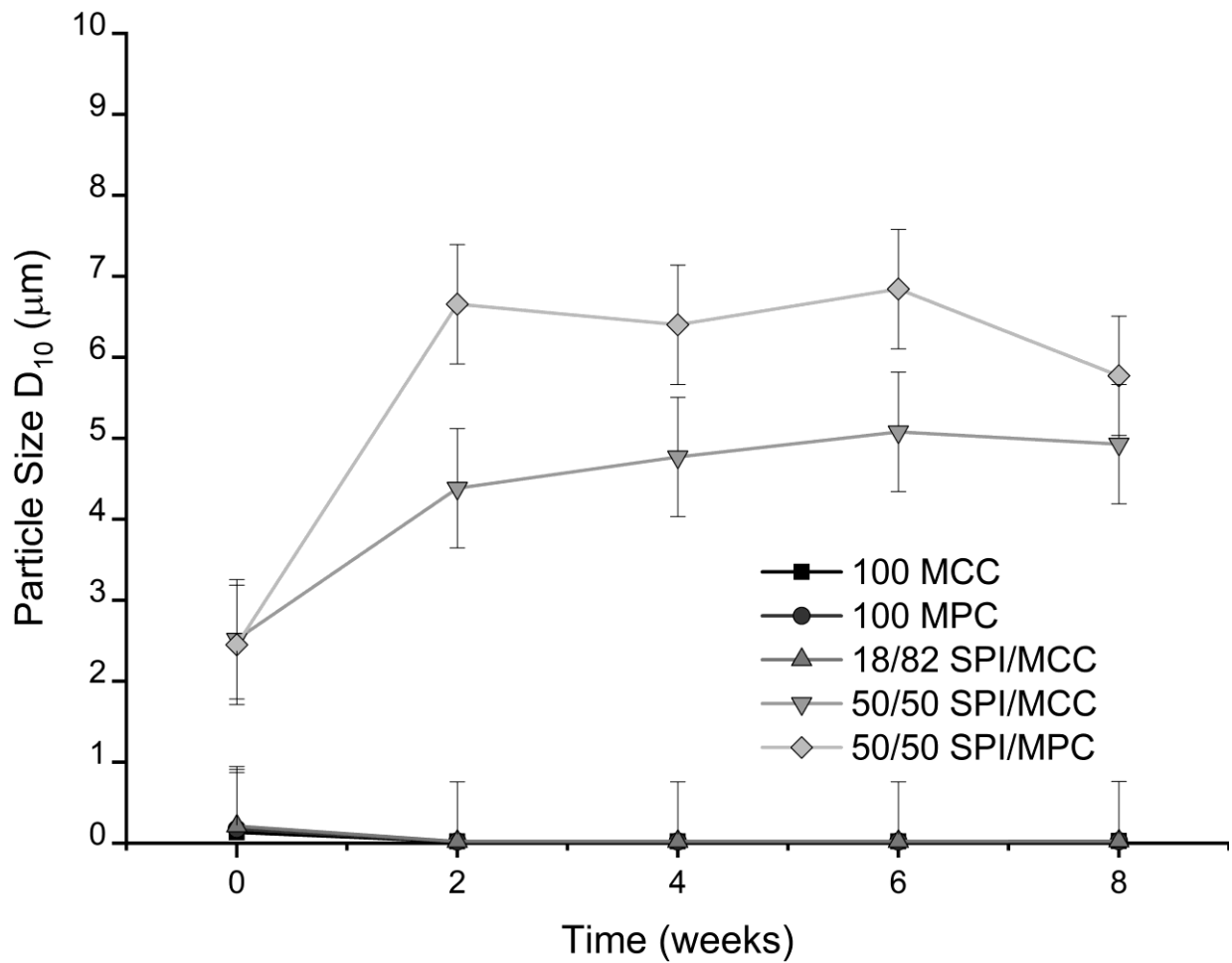


Figure 2.19. Least square means of the particle size  $D_{10}$  ( $\mu\text{m}$ ) of vanilla flavored protein beverages from five different protein blends (trt) at 6.3% protein (Table 2.1) across 8 weeks storage at  $4^{\circ}\text{C}$  (time). Standard error 0.737.



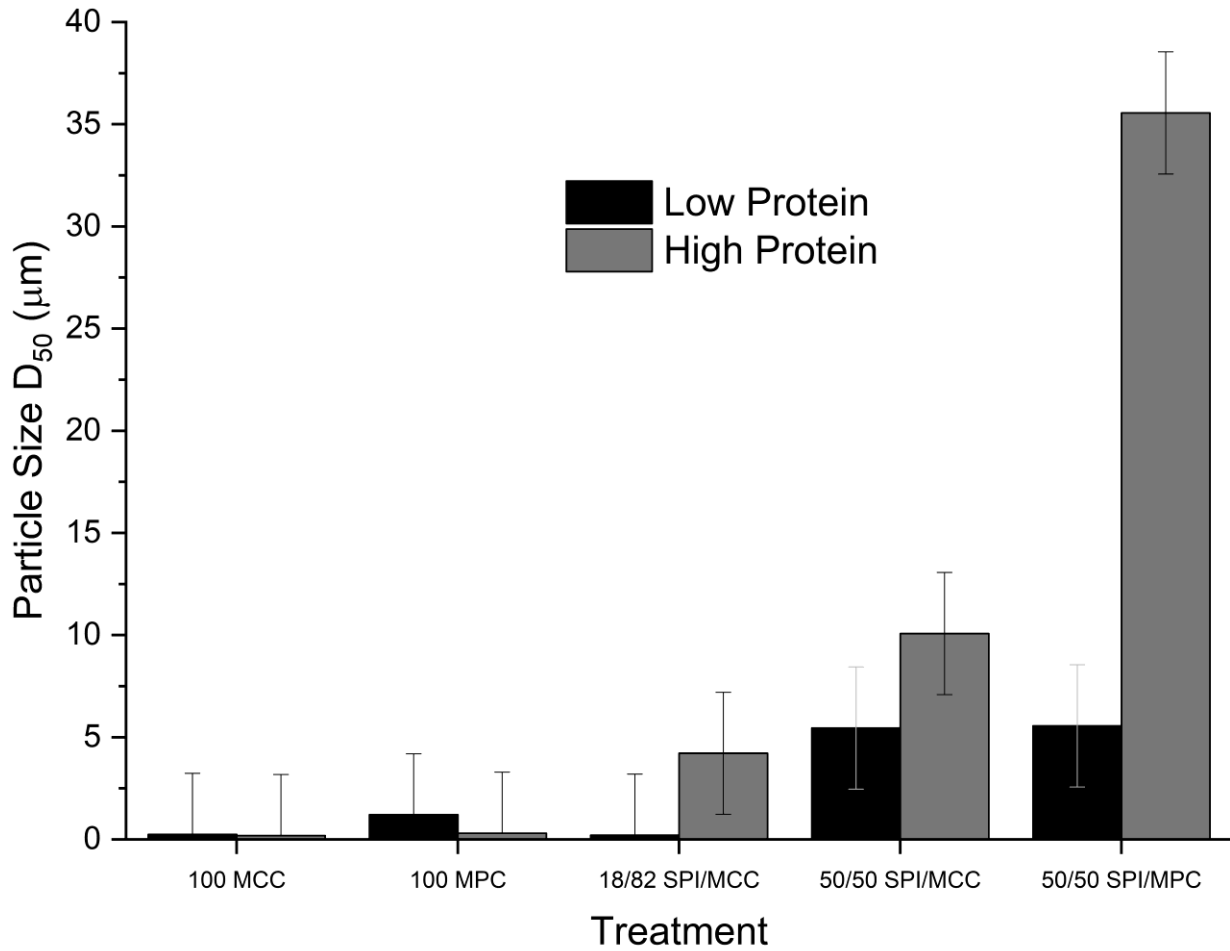


Figure 2.20. Least square means of the particle size  $D_{50}$  ( $\mu\text{m}$ ) of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. There was an interaction (trt x prot) ( $P < 0.05$ ). Error bars above each bar indicate standard error of 2.989.

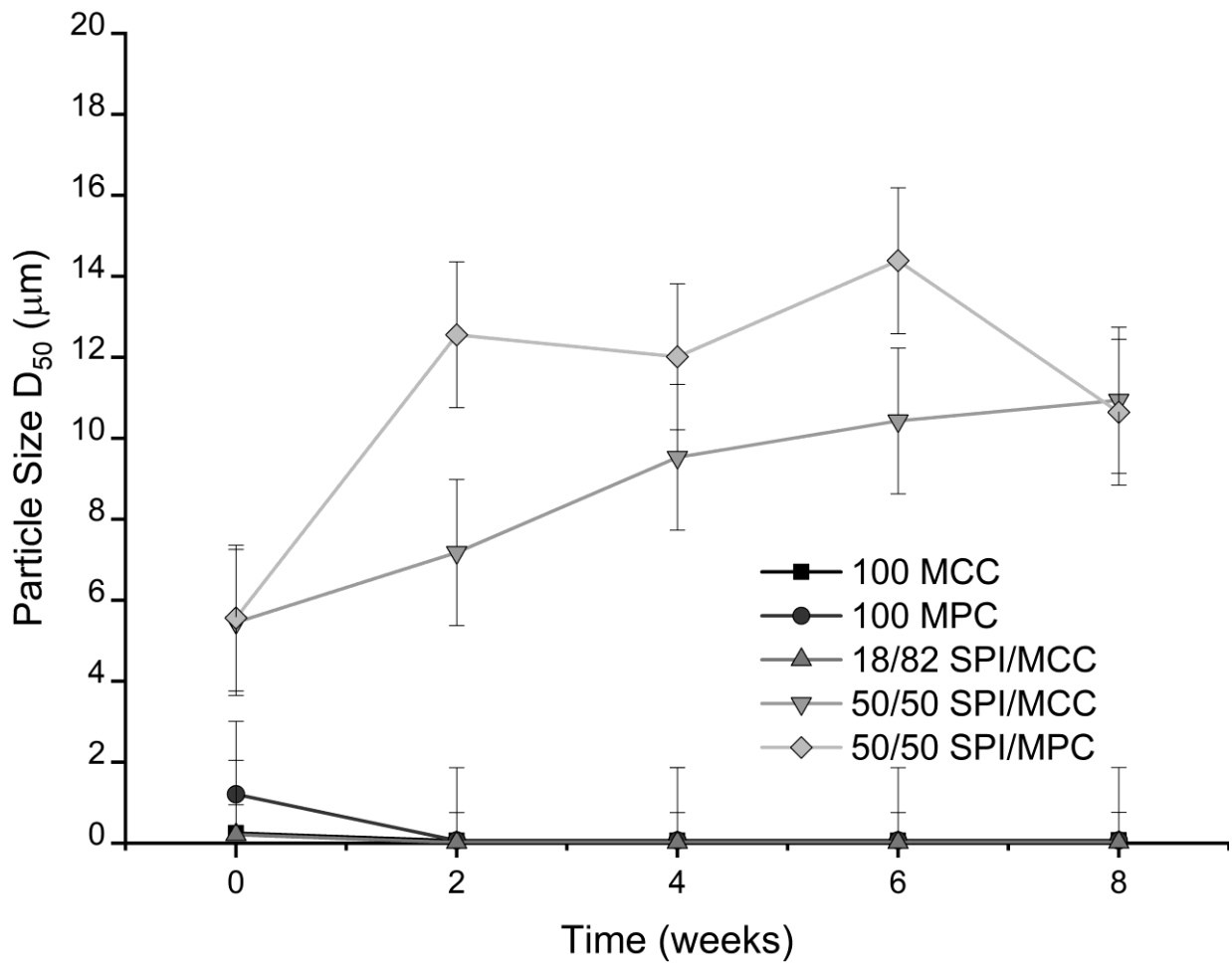


Figure 2.21. Least square means of the particle size D<sub>50</sub> (µm) of vanilla flavored protein beverages from five different protein blends (trt) at 6.3% protein (Table 2.1) across 8 weeks storage at 4°C (time). Standard error 1.801.

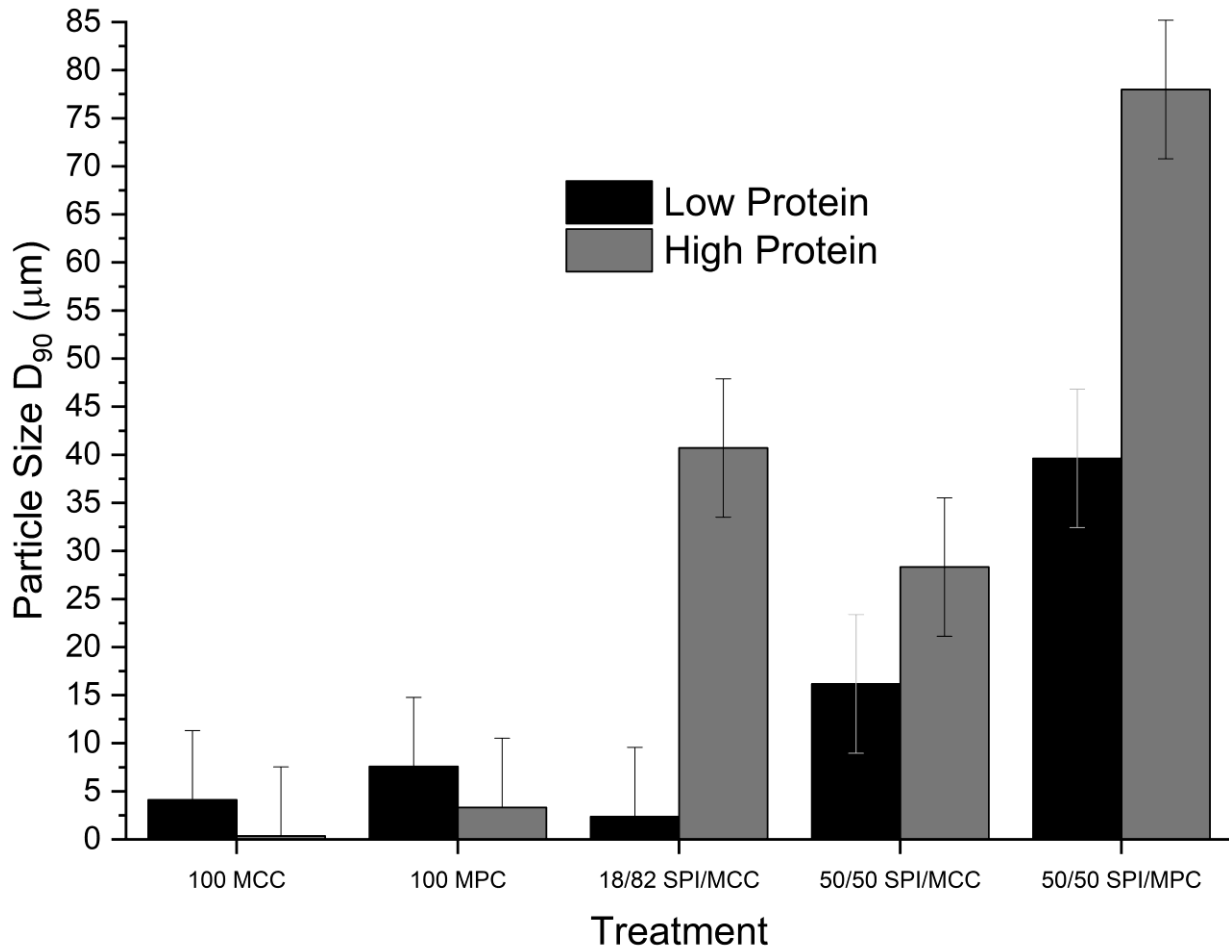


Figure 2.22. Least square means of the particle size  $D_{90}$  ( $\mu\text{m}$ ) of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Error bars above each bar indicate standard error of 7.202.

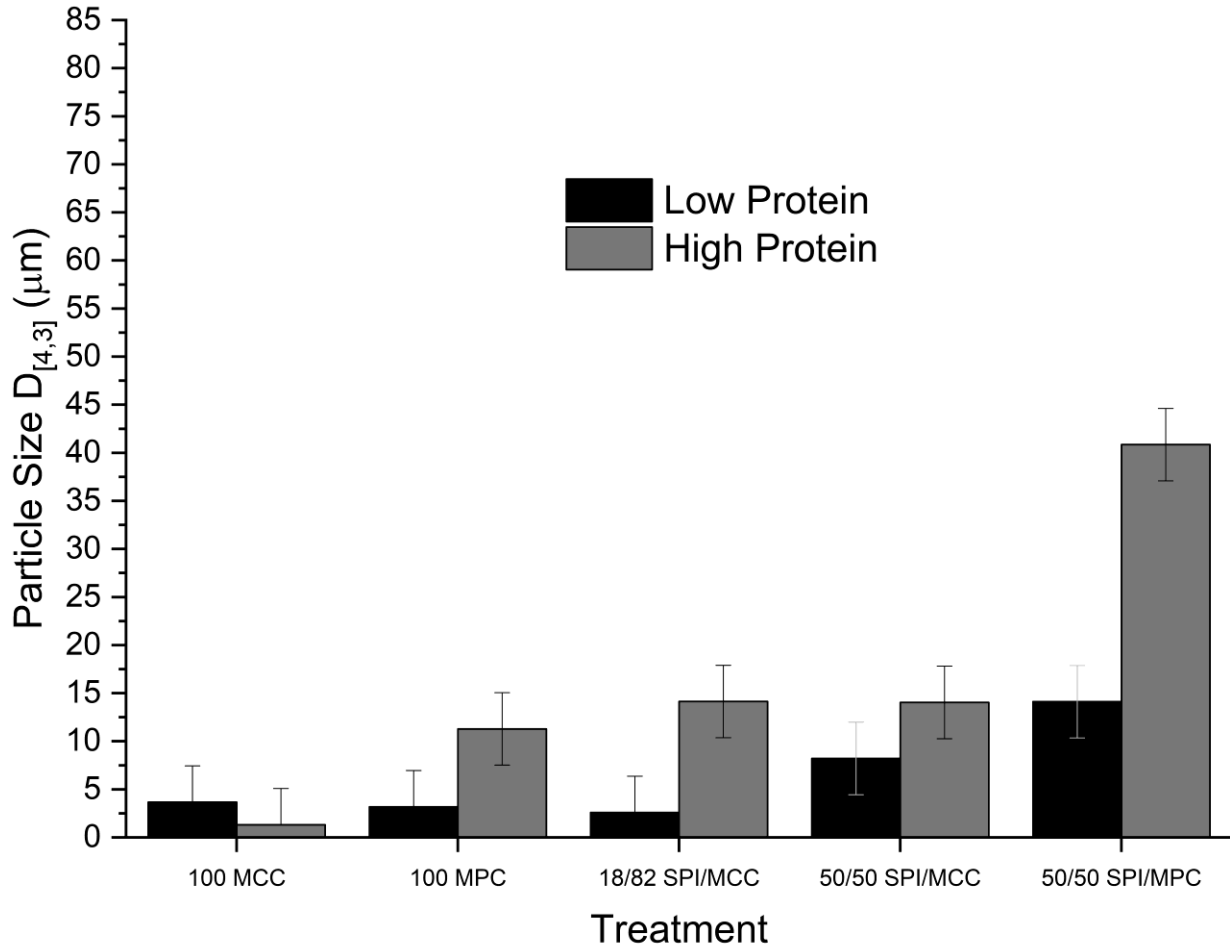


Figure 2.23. Least square means of the particle size  $D_{[4,3]}$  ( $\mu\text{m}$ ) of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. The main effect (trt) was significant ( $P < 0.05$ ) while the main effect (prot) was not ( $P > 0.05$ ). Error bars above each bar indicate a standard error 3.7706.

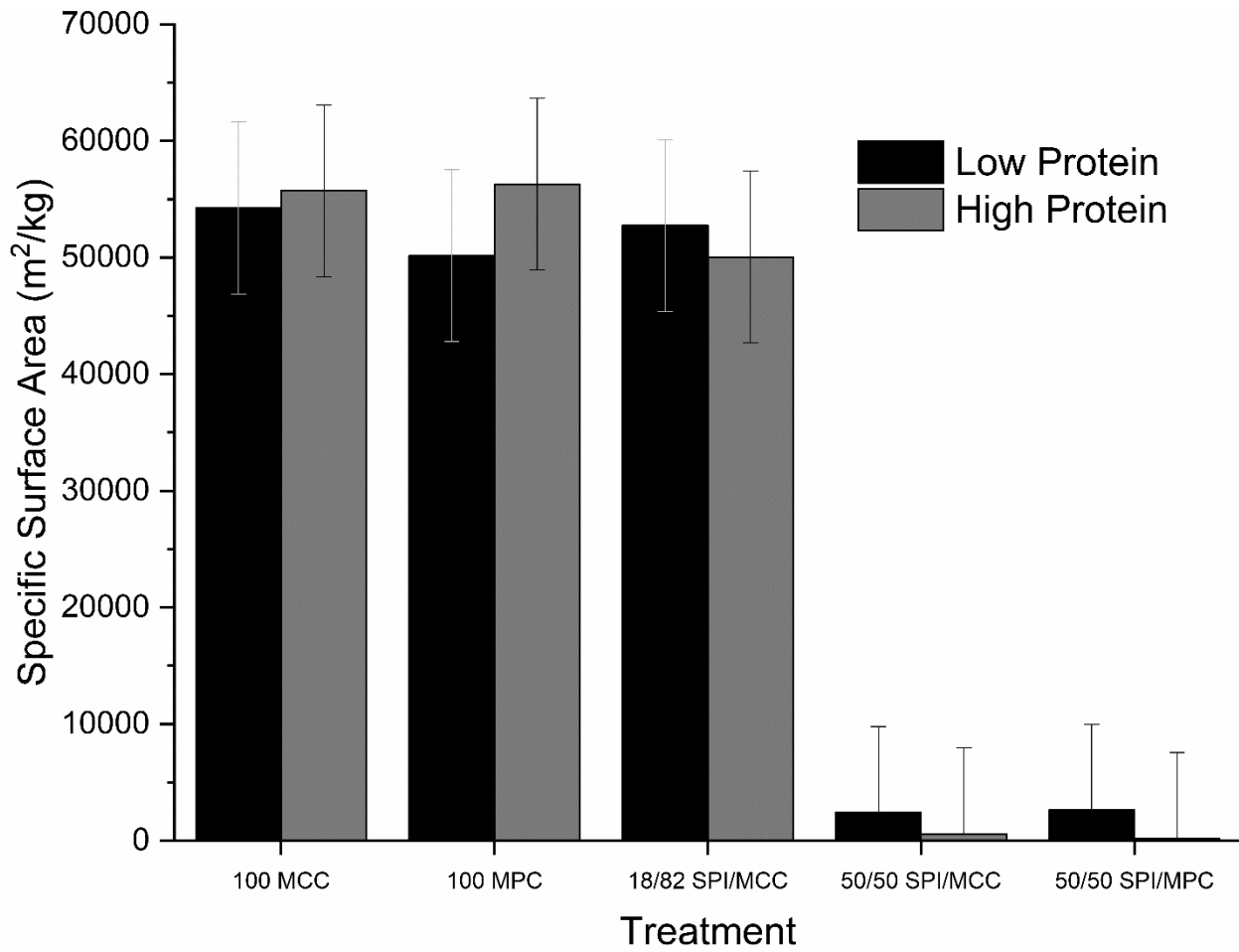


Figure 2.24. Least square means of the specific surface area (m<sup>2</sup>/kg) of the particles in vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) at time 0. Error bars above each bar indicate a standard error of 7368.

## APPENDICES

## APPENDIX A: ADDITIONAL METHODS

### *Color Analysis*

Beverages were tested in duplicate at each time point using an Ultra Scan Pro Spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA). Samples were heated to 20°C in a water bath and measured in reflectance mode at a 5 nm resolution over the wavelength range of 360 to 750 nm, using Illuminant A and a 10° viewer angle. Data was collected for the Hunter L, a, and b values as well as the CIE L\*, a\*, and b\* values, however based on previous work, Hunter L, a, and CIE b\* were reported (Cheng et al., 2018).

### *Apparent Viscosity*

The beverages were tested in duplicate at each time point using a rotational viscometer (LV-DV2T, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) equipped with a jacketed cup and bob fixture (Enhanced UL Adapter, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) using a method adapted from Adams et al. (2015). Samples were measured at 4 and 20 ± 1°C at a constant shear rate of 73 s<sup>-1</sup>. Sixteen (16) mL of tempered sample were added into the cup and water was circulated through the jacket at either 4 or 20°C during measurements. Each sample measurement reflected the average viscosity obtained under 30 s of shear after a 20 s shear equilibration period. Samples that were visibly gelled prior to measurement were excluded as a cup and bob rotational viscometer is unable to adequately describe the more complex rheological properties of a structured gel system (Foegeding et al., 2010).

APPENDIX B: ADDITIONAL DATA

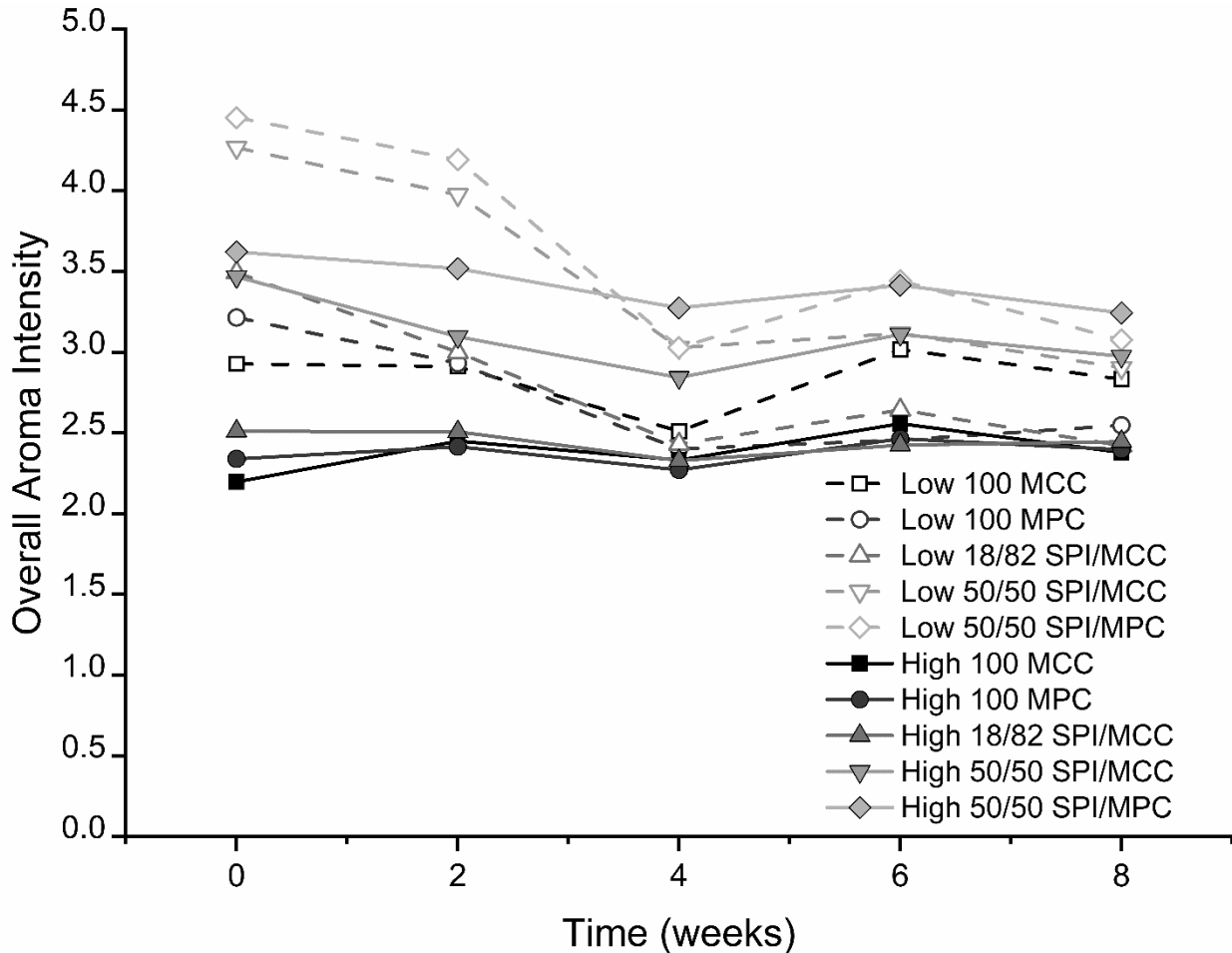


Figure A.1. Least square means of trained panel sensory perception of overall aroma intensity of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. Standard error 0.430.



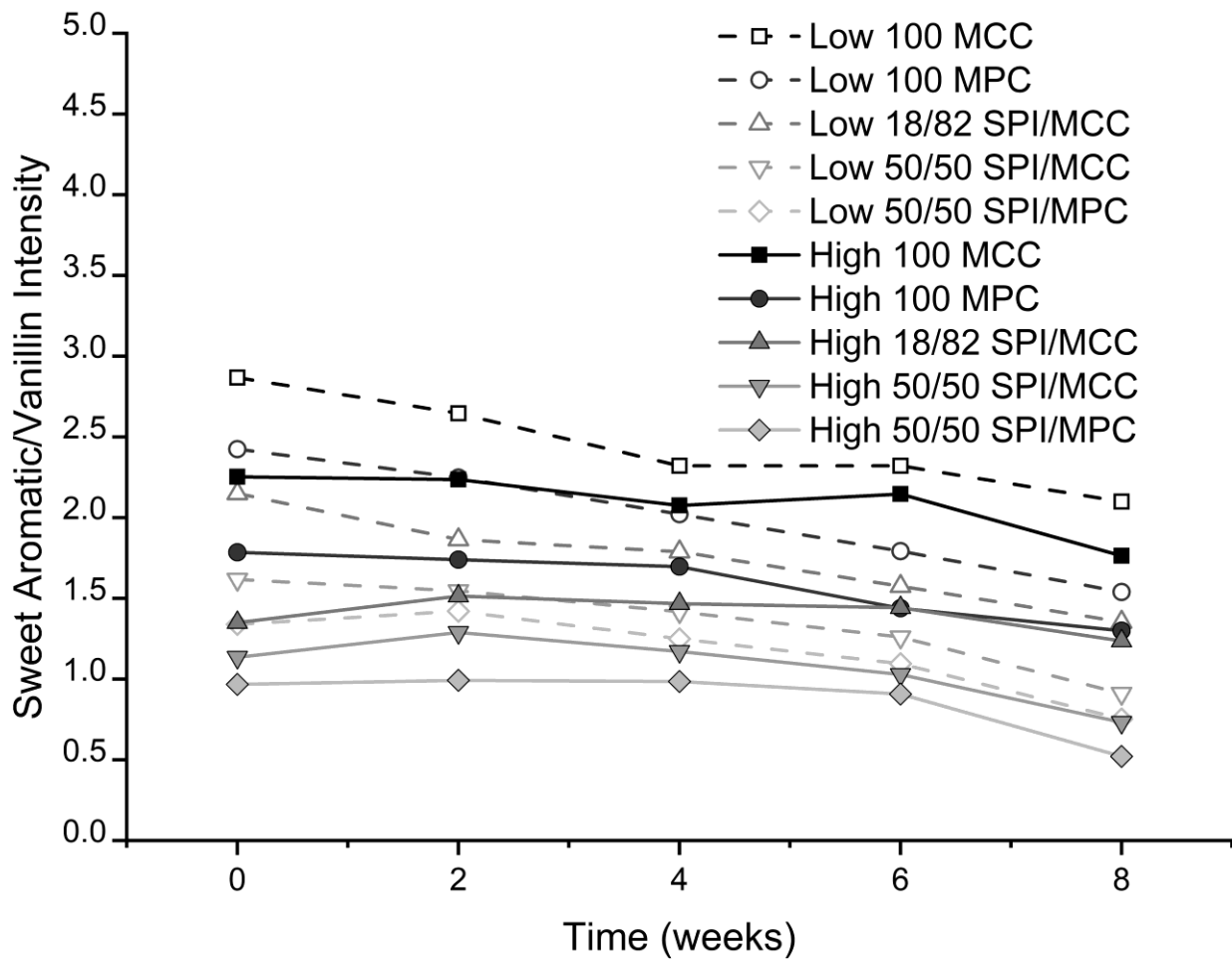


Figure A.2. Least square means of trained panel sensory perception of sweet aromatic/vanillin flavor of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. Standard error 0.154.

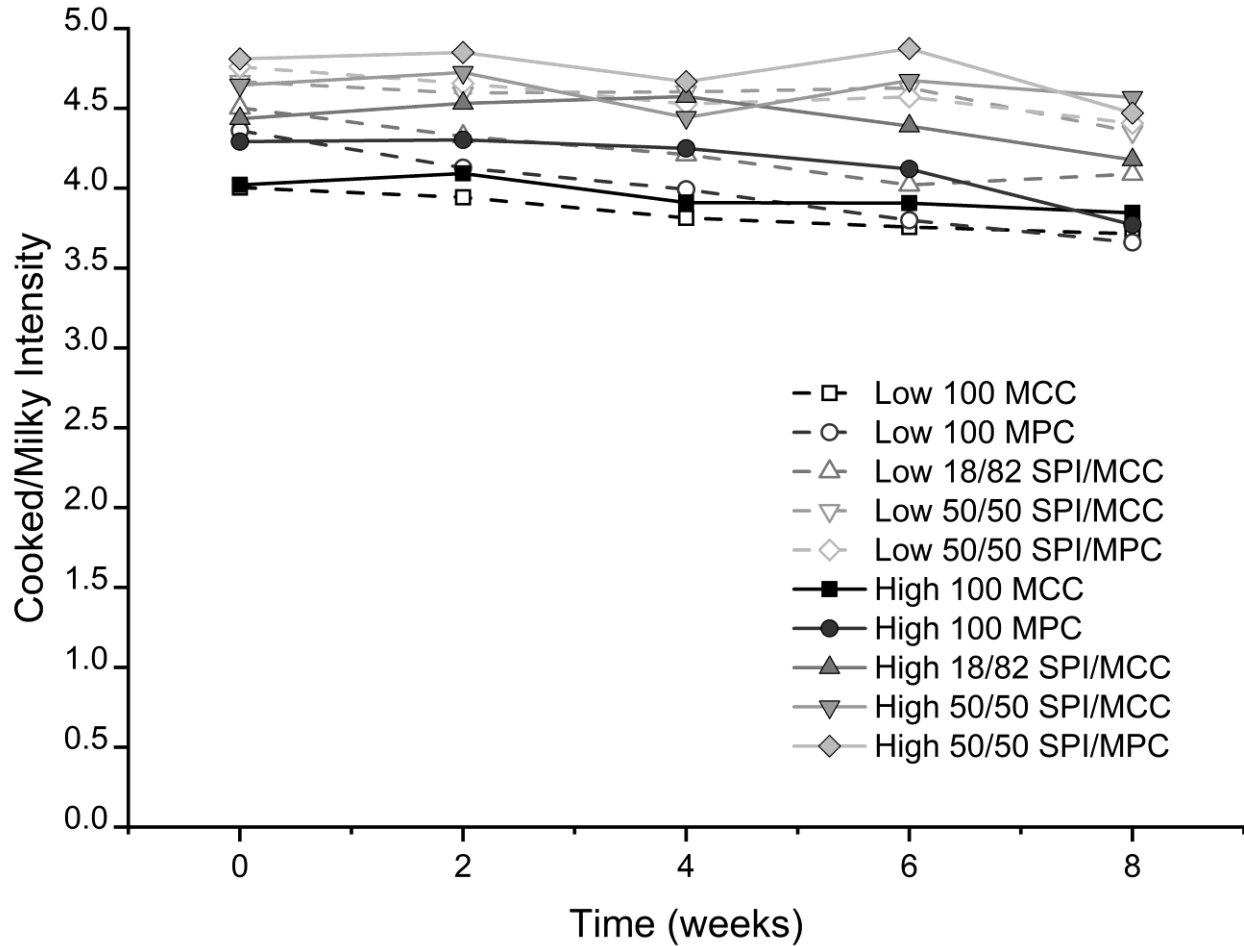


Figure A.3. Least square means of trained panel sensory perception of cooked/milky flavor of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. Standard error 0.102.

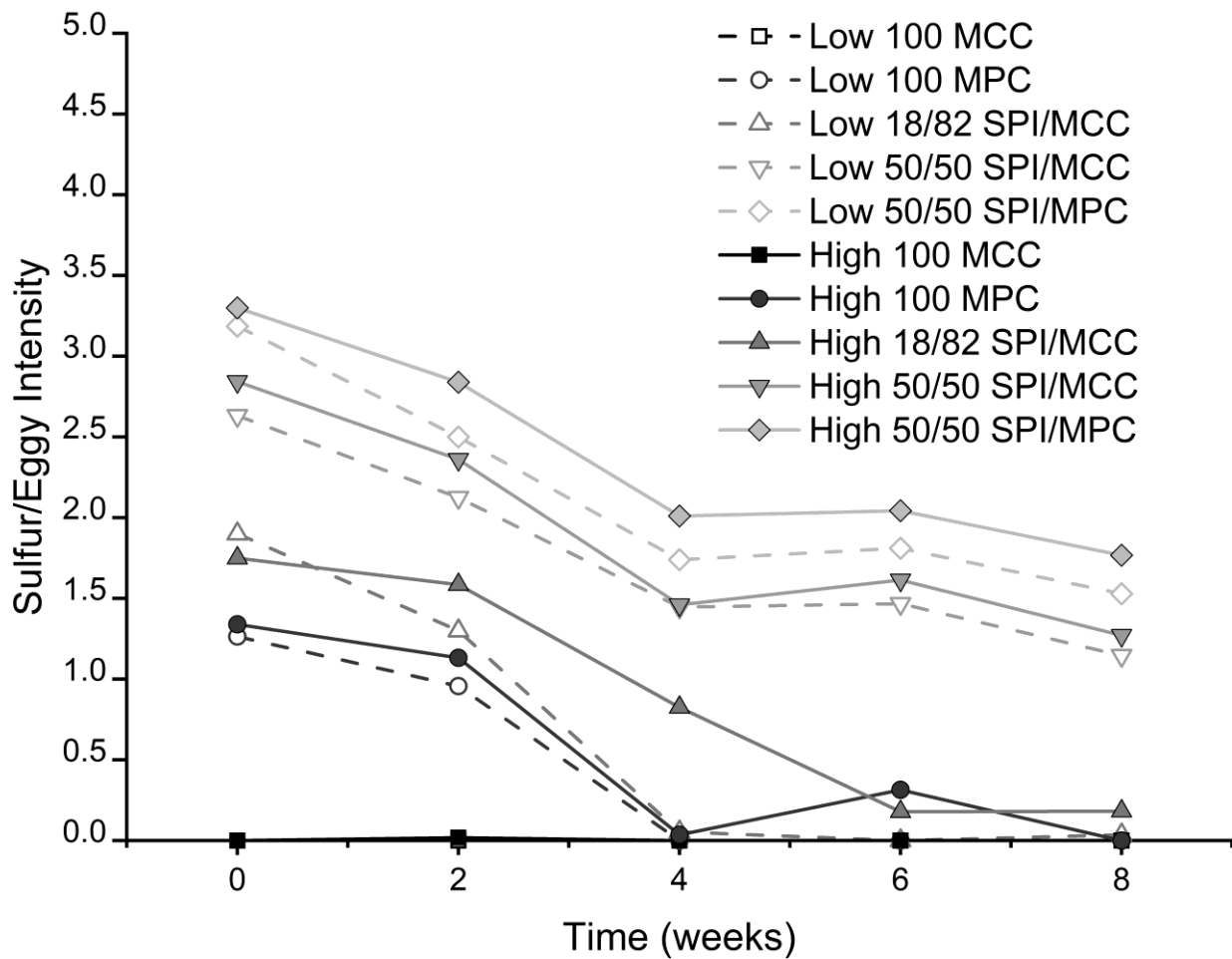


Figure A.4. Least square means of the sensory profiles of sulfur/eggy intensity of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time).. Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. Standard error 0.163.

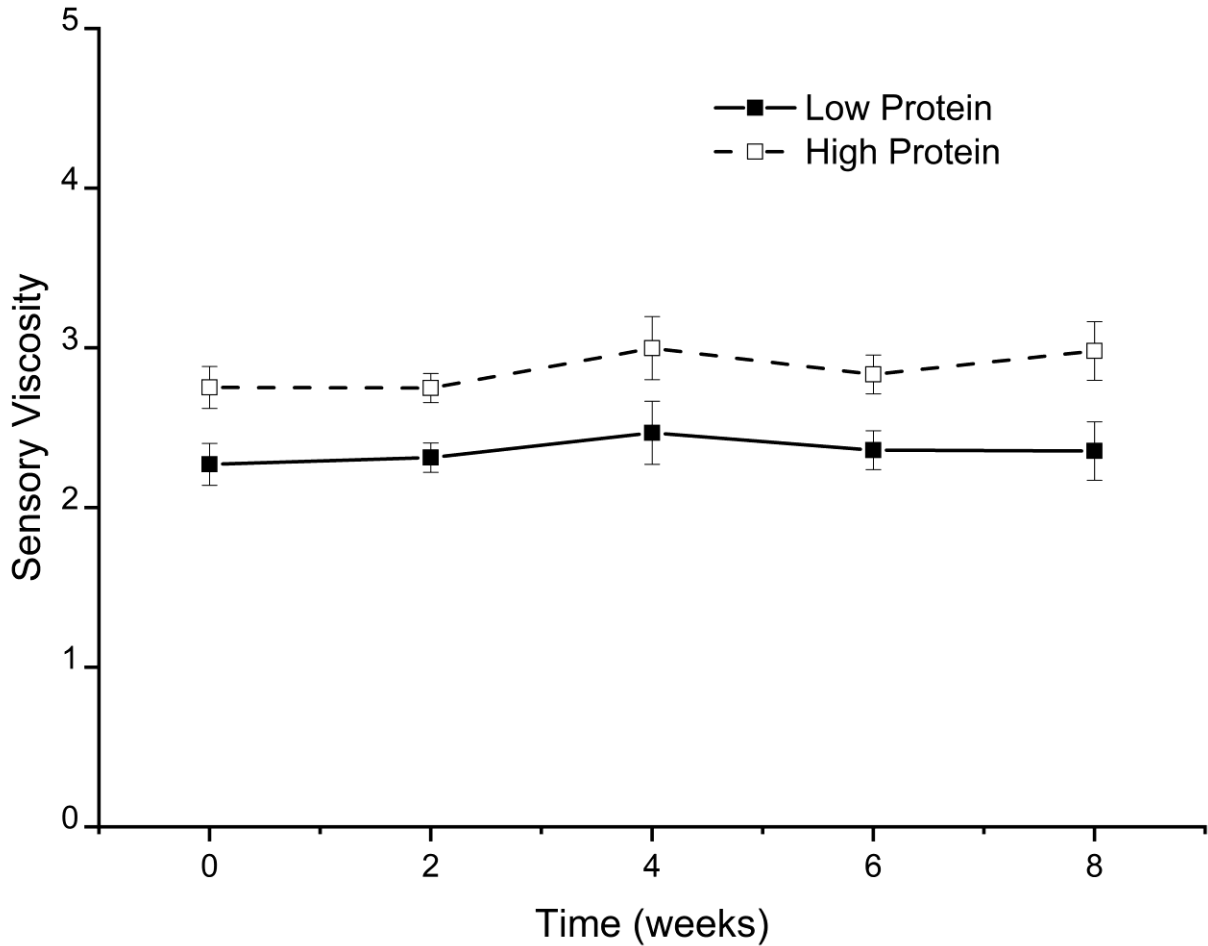


Figure A.5. Least square means of the sensory profiles of sensory viscosity of vanilla flavored protein beverages from five different protein blends (trt) and two protein concentrations (6.3 and 10.5%) (Table 2.1) across 8 weeks of storage at 4°C (time). Sensory attributes were scaled on a 0 to 15 point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Jo et al., 2018; Lee et al., 2017). Graphed with a maximum axis value of 5 to demonstrate differences among beverages.